INTERACTIONS OF PROSTAGLANDINS WITH ADRENERGIC NEUROTRANSMISSION IN THE KIDNEY

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DEDICATION

This thesis is dedicated to my parents for their kind support and encouragement and to Ken for his patience and invaluable help throughout its preparation.

DECLARATION

I declare that this thesis was written by myself and that the research presented therein is my original work. Any assistance with experimental work has been acknowledged.

CONTENTS

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SUMMARY

Considerable evidence has accumulated in recent years which suggests that endogenous prostaglandins, particularly of the E-series, might modulate the release of noradrenaline from peripheral adrenergic nerve endings. Exogenous PGE has been shown to reduce the amount of noradrenaline released from several tissues during adrenergic nerve stimulation. In addition, adrenergic nerve stimulation has been shown to release or increase the release of prostaglandins from several tissues.

The rabbit kidney synthesises large amounts of prostaglandin (mainly PGE_2 and $PGF_{2\alpha}$) and renal nerve stimulation has been shown to increase the output of prostaglandins into renal venous blood. Prostaglandins are known to influence total renal blood flow and its distribution and it is thought that their endogenous production might contribute to renal autoregulation.

The experiments in this thesis were conducted with a view to finding out whether prostaglandins produce their effects on renal blood flow by acting presynaptically to alter vascular tone or by a postsynaptic action.

In Section 1, experiments on rabbits *in vivo* showed, using two different methods of analysis, that noradrenaline, like renal nerve stimulation, increased prostaglandin release from the kidney. It seems likely that the source of the prostaglandin was the postjunctional effector cell rather than the adrenergic nerve ending itself.

In Section 2, experiments were performed on rabbit kidneys *in situ* and *in vitro* to investigate the effects of prostaglandins A_2, E_2 and $F_{2\alpha}$ on vascular responsiveness to injected noradrenaline and to noradrenaline released during renal nerve stimulation. PGA_2 and $PGF_{2\alpha}$ both potentiated vascular responses to noradrenaline and to renal nerve stimulation similarly. It was concluded that their action was primarily at a postjunctional site. PGE_2 inhibited vascular responses to renal nerve stimulation but did not inhibit responses to noradrenaline in parallel, suggesting that it might possess a prejunctional component of action.

In Section 3, the prejunctional action of each prostaglandin on noradrenaline release during RNS was investigated in the rabbit kidney, *in vitro*. All three prostaglandins inhibited noradrenaline overflow from the kidney during renal nerve stimulation but PGE_2 was ten times more potent than PGA_2 or $PGF_{2\alpha}$.

In Section 4, the role of endogenous prostaglandins in the control of adrenergic transmitter release was investigated *in vitro*, using the isolated, perfused rabbit kidney. An attempt was made to reduce prostaglandin synthesis with indomethacin and to increase synthesis with arachidonate. Infusion of indomethacin increased whilst arachidonate infusion reduced the overflow of noradrenaline during renal nerve stimulation.

Prostaglandins released from the kidney during renal nerve stimulation appear to come from postsynaptic sites.

The action of PGE_2 on renal vascular responses to nerve stimulation is, in part, attributable to an action on adrenergic neurotransmission. The evidence which is presented is compatible with the hypothesis that PGE_2 which is released during renal nerve stimulation may, under physiological conditions, reduce the output of noradrenaline from adrenergic nerve terminals during continued nerve stimulation.

GENERAL INTRODUCTION

In 1965 Lee, Covino, Takman and Smith described the isolation of three vasodilator lipids from rabbit renal medulla, one of which was tentatively identified as prostaglandin F (PGF). The second was believed to be PGE and the third, which was inactive on non-vascular smooth muscle, was named "medullin" (Lee et al., 1965). Medullin was subsequently identified as PGA_2 (Lee, Gougoutas, Takman, Daniels, Grostic, Pike, Hinman and Muirhead, 1966). Prostaglandins of the A series, unlike those of the E and F series, are not readily metabolised on passage through the lungs (Horton and Jones, 1969; McGiff, Terragno, Strand, Lee, Lonigro and Ng, 1969). This property, in addition to its ability to lower blood pressure by decreasing total peripheral resistance, appears to fit PGA_2 for the role of a circulating antihypertensive hormone. Furthermore, PGA_2 showed no effect on cardiac contractility or heart rate (Lee et al., 1965). PGE_2 shared with PGA_2 the ability to decrease peripheral vascular resistance and also had similar effects on the renal system (for a comparison of their effects, see Table a). However, its substantial degradation on circulation through the lungs would seem to rule out a role for PGE_2 as a circulating hormone. Thus PGA_2 seems better fitted to the role of a circulating antihypertensive agent. The existence of such a natural hormone was soon challenged, however, when evidence appeared in the literature that PGA was readily formed from PGE by acid treatment, a procedure which was involved during extraction procedures (Strong, Boucher,

Table a: A comparison of the cardiovascular effects of PGA and PGE administered intravenously to the normotensive dog. (Adapted from Lee, 1972).

Target	PGA	PGE
Blood pressure	Lowered	Lowered
Peripheral arterioles	Dilated	Dilated
Total peripheral resistance	Lowered	Lowered
Heart rate	Reflexly increased	Reflexly increased
Myocardial contractility	Unchanged	Unchanged
Renal medullary blood flow	Decreased	Decreased
Renal cortical blood flow	Increased	Increased
Sodium and potassium excretion	Increased	Increased
Urine flow	Increased	Increased
Plasma volume	Decreased	Decreased

PGE was given i.v. in large amounts allowing part to escape degradation by the lung and act systemically. When given in equimolar amounts at which PGA has an intravenous effect ($< 10^{-6}$M), PGE is devoid of systemic circulating actions as a result of its metabolism by the lung.

Nowaczynski and Genest, 1966). It was suggested that PGA might not be naturally-occurring as first thought but rather an extraction artefact. This possibility was further documented by Daniels, Hinman, Leach and Muirhead (1967) who found, by mass spectrometric analysis, that PGE_2 was the principal vasodilator lipid of the rabbit renal medulla. Recent development of more sophisticated and sensitive analytical techniques using isotope dilution overwhelmingly supports the proposition that PGA isolated from the kidney derives solely from dehydration of PGE. Frölich, Sweetman, Carr and Oates (1975) have shown using the deuterium carrier method (cf. Section 1) that all PGA_2 recovered either prior to or following incubation of rabbit renal medullary tissue could be accounted for by in vitro dehydration of PGE_2. Larsson and Änggård (1976) have similarly demonstrated, by use of deuterium and radioactively-labelled prostaglandin internal standards, that the small amount of PGA_2 found in their study of rabbit renal cortex and medulla could be accounted for by conversion from PGE_2.

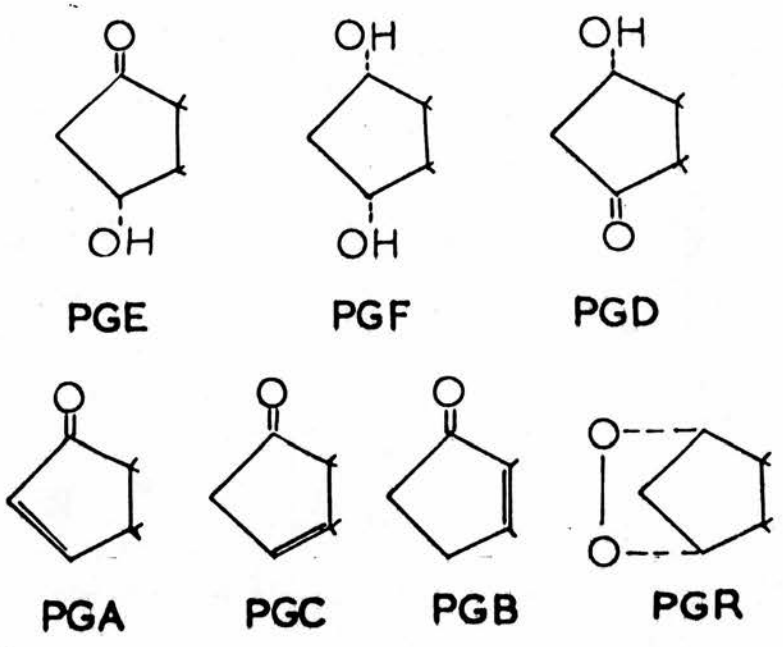
These last authors have shown by the same method that the renal cortex produces approximately equal amounts of PGE_2 and $PGF_{2\alpha}$ and that the renal medulla produces approximately 50% more PGE_2 than $PGF_{2\alpha}$. The functional significance of $PGF_{2\alpha}$ in the kidney is not clear. It produces vasoconstriction in rabbit and rat kidneys (Malik and McGiff, 1975) but in doses comparable to those at which PGE_2 is active, $PGF_{2\alpha}$ does not affect total renal blood flow or its distribution in the dog kidney (McGiff, Terragno, Malik and Lonigro, 1972a; Chang, Splawinski, Oates and Nies, 1975).

The probable artefactual nature of PGA_2 and the general lack of potency of $PGF_{2\alpha}$ in the kidney shed doubt on the possible functions of these prostaglandins as intrarenal hormones. The rapid degradability of PGE_2 on passage through the lung detracts from its suitability as a circulating hormone. It does, however, possess fivefold the vasodilator-diuretic potency of PGA_2 in the renal bed (Lonigro, Terragno, Malik and McGiff, 1973a) and is, as indicated previously, the major prostaglandin synthesised by the kidney. Thus, if any of the prostaglandins has a role in autoregulation of renal blood flow and in diuresis, PGE_2 seems to be the most likely candidate. There is considerable evidence which indicates that PGE_2 is a local intrarenal hormone (Terragno, Strand, Pacholczyk and McGiff, 1973; Vane and McGiff, 1975).

Prostaglandin biosynthesis and metabolism by the kidney

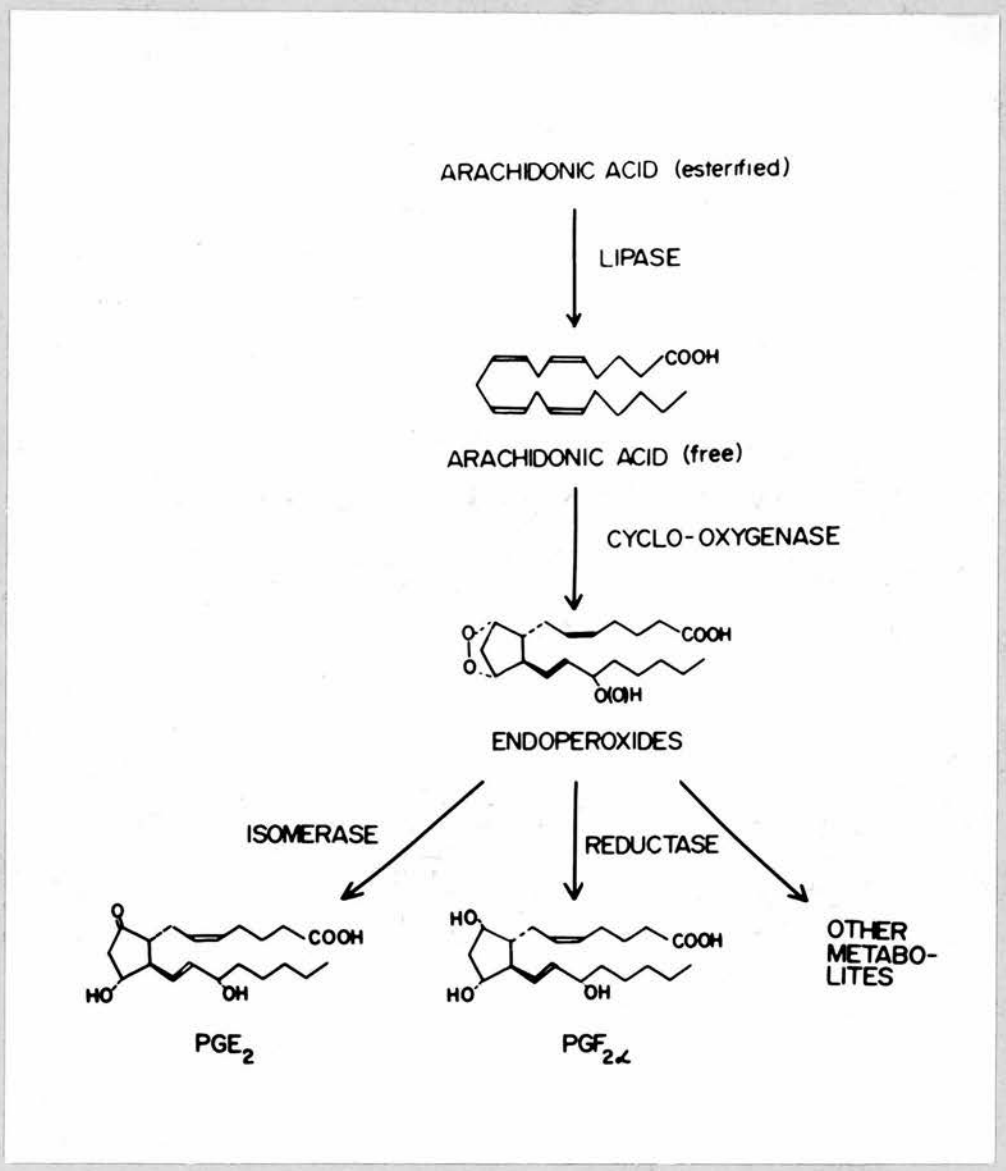
All prostaglandins have as their basic structure a 20-carbon skeleton which has been named prostanoic acid. They are named semi-systematically with reference to this formula (Nugteren, van Dorp, Bergström and Hamberg, 1966). The complexity of their chemical names, however, has led to the adoption of trivial names. Prostaglandins have been divided into groups according to their cyclopentane ring structure, which is denoted by a letter (see Fig. a). The prostaglandins synthesised by the kidney constitute mainly the 2-series, PGE_2 and $PGF_{2\alpha}$. The subscript number refers to the number of double bonds occurring in the two side-chains and α or β to the position of the alcohol groups in

Figure a: Cyclopentane ring structure of each prostaglandin series.



PGF, either both below the ring (α) or 9-OH above and 11-OH below the ring (β). The substrate for the synthesis of these two prostaglandins is the polyenoic fatty acid, arachidonic acid (Bergström, Danielsson and Samuelsson, 1964; van Dorp, Beerthius, Nugteren and Vonkeman, 1964). A scheme of synthesis is represented in Fig. b. Two intermediate endoperoxide compounds have been isolated during enzymatic conversion of arachidonic acid into PGE_2 and $PGF_{2\alpha}$. They were isolated and named simultaneously by Nugteren and Hazelhof (1973) who called them 15-hydroperoxy-PGR_2 and PGR_2 and by Hamberg and Samuelsson (1973), who called them PGG_2 and PGH_2 respectively. PGG_2/15-hydroperoxy-PGR_2 possesses a hydroperoxy (-OOH) group at C_{15} whilst PGH_2/PGR_2 possesses a hydroxyl group at C_{15} (see Fig. b). The prostaglandin endoperoxides have been reported to be more active than the classical prostaglandins in some biological systems e.g. blood platelet aggregation (Hamberg, Hedqvist, Strandberg, Svensson and Samuelsson, 1975). A role for these substances in the kidney, if any, is as yet undiscovered. The endoperoxides can undergo isomerisation to form PGE_2 or PGD_2, or reduction to form $PGF_{2\alpha}$. Another important pathway leads to the formation of major metabolites known as PHD and HHT (Hamberg and Samuelsson, 1974; Hamberg, Svensson and Samuelsson, 1974). It was recently discovered that the intermediate compound between PGG_2 and PHD is also highly active biologically. It has been named thromboxane A_2 as a result of its production by platelets (Hamberg, Svensson and Samuelsson, 1975). This compound is highly labile,

Figure b: Prostaglandin synthesis pathways in the rabbit kidney.



having a half-life of about thirty seconds at 37°C (in aqueous solution). It is now known to be the unstable factor which induces irreversible platelet aggregation when platelets are incubated with either arachidonic acid or PGG_2. There is further evidence which indicates that thromboxane A_2 is also the major component of the rabbit aorta contracting substance (RCS) released from sensitised guinea pig lung during antigen-challenge (Piper and Vane, 1969). PHD belongs to the same group of substances as thromboxane A_2 and has been renamed thromboxane B_2. Thus the thromboxanes constitute a new group of important mediators of the actions of polyenoic fatty acids transformed via the cyclo-oxygenase pathway. Thromboxane production by the kidney has not been investigated as yet and cannot be assumed to occur. Endoperoxide formation, on the other hand, is essential to the production of the renal prostaglandins, PGE_2 and $PGF_{2\alpha}$.

The sites of synthesis and metabolism of prostaglandins in the rabbit kidney can be clearly differentiated. Synthetic capacity appears to increase about tenfold from the renal cortex to the renal medulla, reaching a maximum in the inner medulla or papillary region (Larsson and Änggård, 1973a). Rabbit renal medulla contains one of the most active prostaglandin synthetase enzymes known (Christ and van Dorp, 1972). The cortical synthesis of prostaglandins, although only 10% of that in the medulla, is nevertheless significant in view of the very high synthetic capacity of the medulla. As much as 44 µg PG/g medullary tissue has been

shown to be synthesised (Änggård, Bohman, Griffin, Larsson and Maunsbach, 1972). Recent results suggest that the subcellular location of prostaglandin synthetase is in the endoplasmic reticulum of the medullary cells (Bohman and Larsson, 1975).

Prostaglandins are metabolised in the kidney by conversion of the 15-hydroxyl group to a ketone and then reduction of the 13-14 double bond (Änggård and Larsson, 1971). The enzymes responsible for these transfigurations are 15-hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13}-reductase respectively (Änggård, Larsson and Samuelsson, 1971). In contrast to the distribition of the prostaglandin synthetic enzyme system in the kidney, prostaglandin dehydrogenase occurs most prevalently in the renal cortex, activity in this region being about ten times higher than that found in the outer medulla and papilla (Larsson and Änggård, 1973a). As a result of the high metabolic capacity of the cortex, prostaglandin production in this region remained undetected in earlier studies (Crowshaw and Szlyk, 1970; Crowshaw, 1971). The subcellular localisation of dehydrogenase was found to be the non-particulate fraction of both cortex and outer medulla with no demonstrable activity in the papillary region (Änggård et al., 1971).

The implications of the regionalisation of renal prostaglandin production and degradation are discussed with relation to renal blood flow in the General Discussion at the end of this thesis.

Release of prostaglandins by the kidney

Prostaglandins are released from the kidney in response to a variety of stimuli, all of which involve haemodynamic changes within the organ. Some examples are listed in Table b. Prostaglandin release following vasoconstriction in some of these studies was associated with a recovery or "escape" from vasoconstriction. Prostaglandins have also been implicated as mediators of natriuresis (see Lee, 1972) although these is some disagreement as to whether their effect in promoting salt excretion is the result of haemodynamic changes or of a direct action on renal tubules (Vander, 1968; McGiff et al., 1972a; Gross and Bartter, 1973; Tannenbaum, Splawinski, Oates and Nies, 1975; Slick, Aguilera, Zambraski, Di Bona and Kaloyanides, 1975). The present study is primarily concerned with interactions between prostaglandins and the renal adrenergic system. The major discussion will be confined to aspects of these interactions relating to haemodynamic changes in the kidney.

Renal innervation and vascular anatomy

The renal nerves are mainly adrenergic with fibres originating from the inferior mesenteric ganglion. They form a plexus around the renal artery and pass, in association with it, into the kidney. Adrenergic innervation is associated only with the blood vessels and supplies the main interlobar arteries and the preglomerular arterioles of rabbit, rat and dog kidneys (Nilsson, 1965; McKenna and Angelakos, 1968). In a series of experiments on dog, guinea

Table b: Release of prostaglandins by the kidney

Species	Stimulus	Reference
dog	renal nerve stimulation	Dunham & Zimmerman, 1970.
rabbit	" " "	Davis & Horton, 1972.
dog	noradrenaline infusion	McGiff, Crowshaw, Terragno, Malik & Lonigro, 1972.
cat	" "	Fujimoto & Lockett, 1970.
rabbit	" "	Needleman, Douglas, Jakschik, Stoecklein & Johnson, 1974.
dog	angiotensin II infusion	McGiff, Crowshaw, Terragno & Lonigro, 1970a.
	" "	Needleman, Kauffman, Douglas, Johnson & Marshall, 1973.
dog	renal ischaemia	McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee & Ng, 1970.
dog	reduction in renal perfusion pressure	Sweet, Kadowitz & Brody, 1971.
"	" " "	Sweet, Kadowitz, Forker & Brody, 1972.
"	" " "	Herbaczynska-Cedro & Vane, 1973.
dog	reduction in renal blood flow	Herbaczynska-Cedro & Vane, 1973, 1974.
dog	endotoxin-induced hypotension	Collier, Herman & Vane, 1973. Herman & Vane, 1974.

pig, rat, hamster and mouse kidneys, Dolezel (1966) also demonstrated adrenergic innervation of the large veins. The overall distribution of renal innervation suggests that it may have an integral role in the control of renal blood flow.

The large interlobar arteries arising from the main renal artery supply glomeruli in both the outer and inner (juxtamedullary) cortex. Each outer cortical glomerulus consists of an afferent arteriole, a glomerular tuft and an efferent arteriole, with no evidence of a connection between the arterioles other than via the glomerulus. The efferent arteriole empties into a network of cortical capillaries of small calibre. The juxtamedullary arteriole-glomerular units of the human and rabbit kidney have been shown to differ from those in the outer cortex (Ljungqvist, 1964). They consist of afferent and efferent arterioles which form continuous vessels and from which glomerular capillaries are given off as side-branches. The efferent part of the continuous vessel empties ultimately into straight vessels of large calibre, known as the vasa recta, which penetrate the medulla (Trueta, Barclay, Daniel, Franklin and Prichard, 1947; Ljungqvist, 1964). In normal kidneys, all blood circulating through the organ passes through the glomeruli and the medulla receives only the blood supply which enters it through the juxtamedullary glomeruli (not more than 25% of total renal blood flow). During intense cortical vaso-constriction, blood is diverted from the cortex to the medulla and filtration is reduced or eliminated. The juxta-medullary vessels are responsible for the by-pass of blood,

the calibre of these vessels controlling the magnitude of the extra-glomerular flow.

Nervous control of renal blood flow

During renal nerve stimulation (RNS), blood is "short-circuited" from the cortex to the medulla via the juxta-medullary shunt described above. Pomeranz, Birtch and Barger (1968) have demonstrated that RNS at 10 impulses/second (10 Hz), which may not alter total renal blood flow, decreases perfusion of the outer cortical capillaries and increases that of the outer medulla. At higher frequencies of stimulation (30-32 Hz), vasoconstriction has been shown to occur in both the outer and juxtamedullary cortex, producing cessation of blood flow through this region, whilst blood in the medulla remains static. A significant shunting of blood flow from cortex to medulla was not observed in these experiments (Block, Wakim and Mann, 1952). The stimulation frequency employed by these authors was, however, far in excess of physiological impulse frequency in renal nerves. In the cat it has been demonstrated that, even when baro-receptor inhibition is completely abolished, the impulse traffic in renal afferent nerves rarely exceeds about 4 Hz (Kendrick, Öberg and Wennergren, 1972). Redistribution of renal blood flow has been demonstrated at higher stimulation frequency, however, using the radioactive microsphere technique. Slick et al. (1975) found that the distribution of renal blood flow to the outer cortex was diminished after RNS at 20 Hz. In summary, RNS alters the distribution as well as the total amount of blood flowing to

the various regions of the kidney. The functional aspects of nervous control of renal blood flow are considered in the General Discussion at the end of this thesis.

Prostaglandins and the adrenergic nervous system

The first interaction between prostaglandins and catecholamines was that noted by Euler in 1939. He found that intravenous injection of prostaglandin extract attenuated pressor responses to adrenaline in the rabbit (von Euler, 1939). In 1963, following purification and identification of prostaglandins, this observation was confirmed using pure PGE_1 (Holmes, Horton and Main, 1963). Furthermore, these authors demonstrated that pressor responses to vasopressin, angiotension and noradrenaline were similarly attenuated by PGE_1. These early observations were succeeded by a large number of studies by other workers in the field who investigated the interactions of prostaglandins with adrenergic stimuli in a variety of vascular and non-vascular smooth muscle preparations (for reviews, see Horton, 1969, 1972, 1973; Hedqvist, 1970a; Weeks, 1972; Brody and Kadowitz, 1974; Kadowitz, Joiner and Hyman, 1975). It was not until 1969, however, that the effect of prostaglandin on adrenergic transmitter release was investigated (Hedqvist, 1969a, 1969b; Hedqvist and Brundin, 1969). These authors demonstrated that close-arterial infusion of PGE_1 into the isolated cat spleen reduced overflow of noradrenaline (NA) in response to splenic nerve stimulation in four out of ten experiments. When PGE_2 was infused in the same concentrations,

however, NA overflow consistently fell, often to less than 50% of the preceding control. Phenoxybenzamine, which has been shown to inhibit the release of prostaglandins from some tissues (see Section 1), when infused into the spleen, resulted in a sevenfold increase in NA overflow in response to nerve stimulation (Hedqvist, 1969b, 1969c). Simultaneous infusion of PGE_2 strongly counteracted this effect. Hedqvist suggested, therefore, that PGE might be locally mobilised on sympathetic nerve stimulation to act as a negative feedback inhibitor of further NA release from adrenergic nerves. He then proceeded to investigate this concept further in cat spleen, rabbit heart and guinea pig vas deferens (Hedqvist, 1970a). In 1971 Swedin, a colleague of Hedqvist, made an important observation which contributed to the idea that endogenous prostaglandin might regulate adrenergic transmitter release. He found that repeated nerve stimulation or field stimulation of the guinea pig vas deferens depressed the initial rapid phase "twitch" of the organ and he made the following observations:

1) inhibition was immediately abolished upon washing the organ
2) inhibition was greater in a small-volume organ bath than in a larger one
3) an inhibitory factor could be transferred from one bath to influence another organ
4) this substance probably acted prejunctionally since tests with exogenous NA revealed unaltered responses.

These observations led the author to the conclusion that inhibition was induced by some substance released together

with the transmitter upon nerve stimulation. That this inhibitory substance was prostaglandin-like was suggested by the following results:

1) the inhibitory agent influenced both phases of contraction (slow and fast) of the vas deferens, as did PGE_1 and PGE_2.

2) incubation of the tissue with the prostaglandin synthesis inhibitor, eicosatetraynoic acid (ETA) abolished inhibition.

The identification of the substance as PGE_2-like was established by thin layer chromatography.

This piece of work (Swedin, 1971) establishes an important link between the effects of exogenous prostaglandins and those of endogenous prostaglandins on adrenergic neurotransmission. These observations in conjunction with those of Hedqvist led, inevitably, to the implication that prostaglandins might owe at least part of their potent action on smooth muscle to an ability to alter sympathetic tone. The discovery of drugs which block the synthesis of prostaglandins (for references, see Section 4) permitted further investigation of the role of endogenous prostaglandin in sympathetic neurotransmission. Such drugs, e.g. indomethacin, have been shown to reduce blood flow in several vascular beds by inducing vasoconstriction (see Vane and McGiff, 1975). It is tempting to speculate, therefore, that these drugs act by reducing the prostaglandin braking mechanism on adrenergic transmitter release, thereby increasing vascular sympathetic tone.

The following aspects of the research described on adrenergic/prostaglandin/renal interactions provided the stimulus for the work presented in this thesis:

(1) RNS increases the output of endogenous prostaglandins from the kidney.

(2) Exogenous prostaglandins can modify adrenergic transmitter release from adrenergic nerve endings.

The experiments described in Sections 1-4 were designed to answer the following questions:

(i) Are prostaglandins released presynaptically from adrenergic nerve endings or postsynaptically from vascular smooth muscle in the kidney?

(ii) Do prostaglandins influence renal vascular responsiveness to both RNS and circulating NA?

(iii) Do prostaglandins owe all or part of their action on renal vasculature to an effect on vascular sympathetic tone, by virtue of their ability to alter adrenergic transmitter release?

(iv) Is <u>endogenous</u> prostaglandin involved in the control of sympathetic transmitter release in the kidney and hence renal vascular tone?

The species chosen for study was the rabbit, as a considerable proportion of research into prostaglandin biosynthesis, release and metabolism has been carried out in rabbit kidney.

SECTION 1

The effects of noradrenaline on prostaglandin output from the rabbit kidney, in vivo and in vitro

The release of prostaglandins from the kidney in response to renal nerve stimulation (RNS) or noradrenaline (NA) is a matter of some dispute. In the dog, it has been demonstrated by Dunham and Zimmerman (1970) that RNS causes the release of a prostaglandin-like material (resembling PGE_2) into the renal venous blood and by Terashima, Anderson, Jubiz, Tsagaris and Kuida (1974) that NA causes an increased output of PGE into renal venous blood. In contrast, McGiff and colleagues have shown a differential effect of these two stimuli, where NA causes an increase in prostaglandin output, but RNS does not (McGiff, Crowshaw, Terragno and Lonigro, 1970a; McGiff, Crowshaw, Terragno, Malik and Lonigro, 1972). The methods used for measurement of prostaglandins in all of the foregoing experiments did not include absolute chemical identification. Davis and Horton (1972) have provided evidence (using combined gas chromatography - mass spectrometry for absolute identification) that RNS increases the output of prostaglandins in renal venous blood of rabbits. As an extension of their work, this section describes a series of experiments on the effect of noradrenaline on output of prostaglandins from the rabbit kidney in vivo.

It is of particular interest to know whether NA, as well as RNS, is capable of releasing prostaglandins from

the kidney since this may, indirectly, indicate the site of origin of released prostaglandins. They may be released either from adrenergic nerve endings, i.e. prejunctionally, from the post-synaptic effector cell, or from both of these sites. If only RNS is capable of releasing prostaglandins, one might expect the source to be prejunctional. If, however, both RNS and NA release prostaglandins, it is likely, although not certain, that the release is from a postjunctional site. Various lines of evidence relating to site of release and its implications are discussed at the end of this section.

A small group of experiments which were carried out at a later date on the isolated kidney, is included in this section. These experiments were conducted to test the viability of the in vitro preparation. They describe the output of prostaglandins in the perfusate under resting conditions and during RNS. For details of the isolated kidney experimental design, the reader is referred to Section 2.

Section 1 describes the utilisation of various techniques for the estimation of prostaglandins. Some of these techniques were developed in the laboratory during the course of the present experiments. The advantages and/or disadvantages of each method are discussed in the methods sub-section.

General Methods

1. Experimental Procedures

(a) Surgical procedure

Male New Zealand white rabbits were injected intravenously with 25% urethane, 7 ml/kg, which produced a good depth of anaesthesia with little reflex muscle activity, thus facilitating dissection procedures.

Two or three donor rabbits (litter-mates to the experimental animal if possible) provided replacement blood for each experimental animal. The donor animals were anaesthetised as above and were given an intravenous injection of heparin, 1 i.u./g. Blood was collected into polythene tubes via a cannula inserted into the abdominal aorta.

In the experimental rabbit, the trachea, right external jugular vein and right femoral vein were cannulated. In addition, a fine catheter was inserted into the right femoral artery, and pushed up into the abdominal aorta until its tip lay just rostral to the renal arteries. Blood pressure was monitored from the left carotid artery using a Statham pressure transducer (P23 DC) connected either to a Grass polygraph or to a Devices recorder. The abdomen was opened along its midline and the renal artery and vein were cleared. Ties were placed round the vein. The abdomen was then closed and kept moist with warm 0.9% saline. The animal was left for about 15 min until capillary bleeding resulting from surgery had lessened or stopped. Heparin (1 i.u/g.) was then injected intravenously via the femoral

vein and the left renal vein was cannulated. The left vein was chosen since it is considerably longer than the right, in the rabbit. The blood supply to the kidney was never cut off for more than 2-3 min during cannulation of the vein. The renal venous cannula was connected to a Y-piece, one arm of which was attached to the external jugular venous cannula and the other arm connected to a side-arm of polythene tubing which allowed for the collection of renal venous blood samples. After cannulation of the renal vein, the side-arm was clamped off and the blood was directed from the kidney to the external jugular vein. Heparinised donor blood (10 ml) was then slowly infused via the femoral vein by a Watson-Marlow pump, to compensate for the exteriorised circuit blood. The abdomen was closed off and the animal was maintained at 37°C by use of a heating mat.

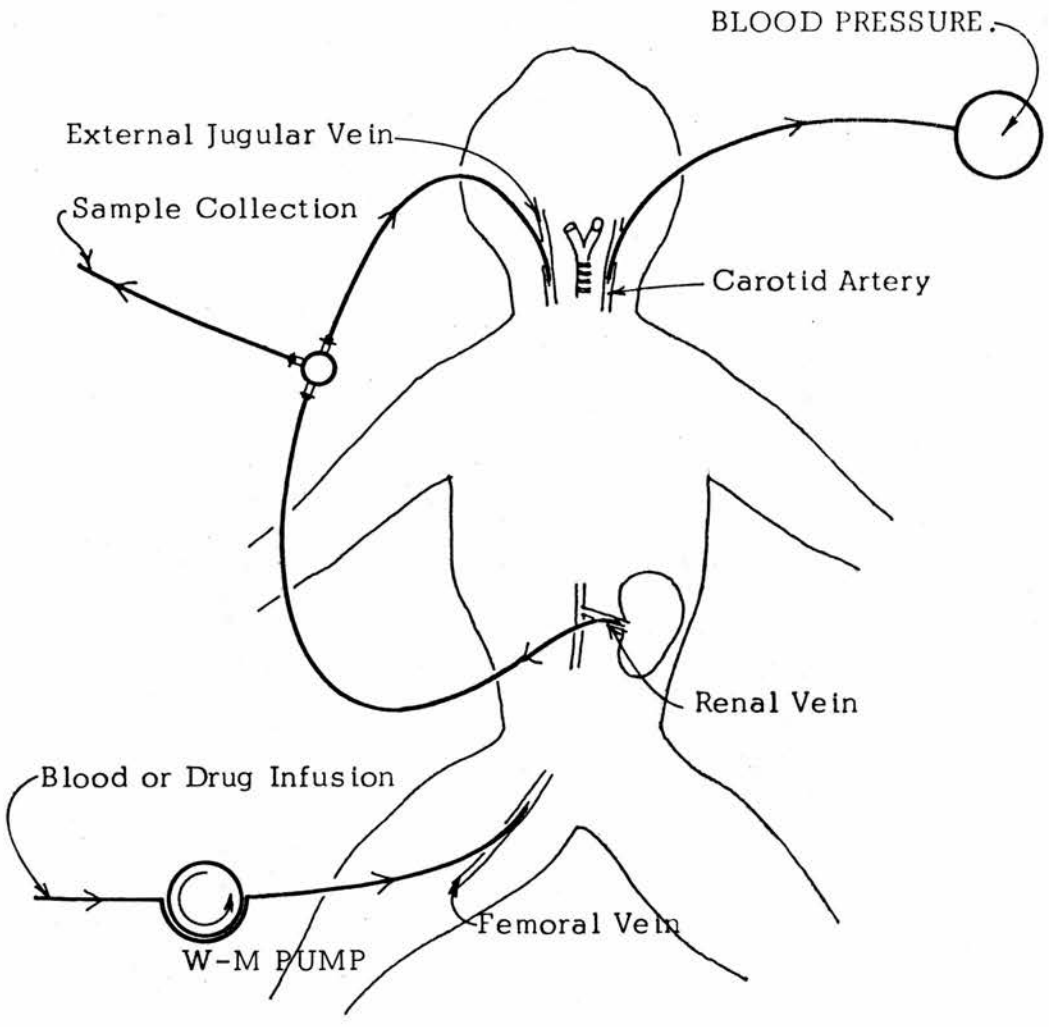
All venous cannulae were filled with 0.9% saline containing 50 i.u./ml heparin and arterial cannulae with saline containing 500 i.u./ml heparin.

A period of 30 min was allowed for the animal to re-gain homeostasis before collecting blood samples. The experimental arrangement is represented diagrammatically in Fig. 1a.

(b) Blood sample collection

Renal venous blood samples were collected by clamping off the external jugular cannula and hence redirecting the blood through the collection side-arm. Measuring cylinders

Figure 1a: Experimental rabbit dissection.



containing 0.1 μCi of each tritiated PGA_1, PGE_2 and $PGF_{2\alpha}$ were used for collection of samples to be analysed by bioassay and combined gas chromatography/mass spectrometry. As indicated in the results sub-section, these samples were collected over periods of 2 to 5 min and ranged in volume from 20 to 50 ml. In later experiments, where the blood samples were assayed for prostaglandin content using radioimmunoassay, collection of small blood samples, each 3 ml, was possible; no radioactive prostaglandins were added to these samples. Exact time intervals between consecutive samples are indicated for each individual experiment in the results sub-section.

The effect of noradrenaline on output of prostaglandins from the kidney was tested by intra-arterial infusion of the drug (Levophed, Winth) via the femoral arterial cannula, which ended just above the renal artery. It was assumed that a proportion of the noradrenaline would be washed into the renal artery by this means. The dose range investigated was 50-1000 ng/kg/min and infusion times were approximately 10 min in duration. Renal venous blood was sampled prior to, during and subsequent to noradrenaline infusion.

2. Assay Procedures

(a) Assay of prostaglandins by bioassay and combined gas-liquid chromatography/mass spectrometry

Plasma Extraction Procedures

Prostaglandin extraction techniques were based on those described by Bergström and Samuelsson (1963) and

Horton and Main (1967). The procedures are outlined in Figs. 1b and 1c. The latter figure describes an additional extraction step used to further purify fractions containing F prostaglandins following thin layer chromatographic separation. PGF is the most polar of the prostaglandins measured and thus remained close to the origin of the chromatographic plate. This region retained many of the pigments of the biological sample with the result that the F-fraction tended to be highly coloured. Further purification involved extraction into phosphate buffer, pH 8.

Blood samples, collected as described in Experimental Procedures and containing tritium labelled PGA_1, PGE_2 and $PGF_{2\alpha}$, were centrifuged at 2,500 rpm for 10 min at 4°C. Plasma was removed using Pasteur pipettes and the cells were washed with 0.9% ice-cold saline (a volume equivalent to that of the original blood sample) and centrifuged once more as described. The supernatant was removed and bulked with the plasma. After measuring the volume, the diluted plasma was acidified to pH 4.5 with 10% acetic acid. The acidified fluid was then partitioned twice with two times an equal volume of redistilled ethyl acetate, the organic phases were pooled and the pH of the aqueous phase was checked. The organic phase was washed with about 1/10 vol H_2O, the water discarded and the ethyl acetate evaporated to dryness in a pear-shaped flask under reduced pressure. The residue was desiccated under vacuum to remove any remaining acetic acid. It was then dissolved in 30 ml 67% ethanol and shaken in a separating funnel with 20 ml heavy

Fig. 1b: Extraction of prostaglandins from renal venous plasma samples

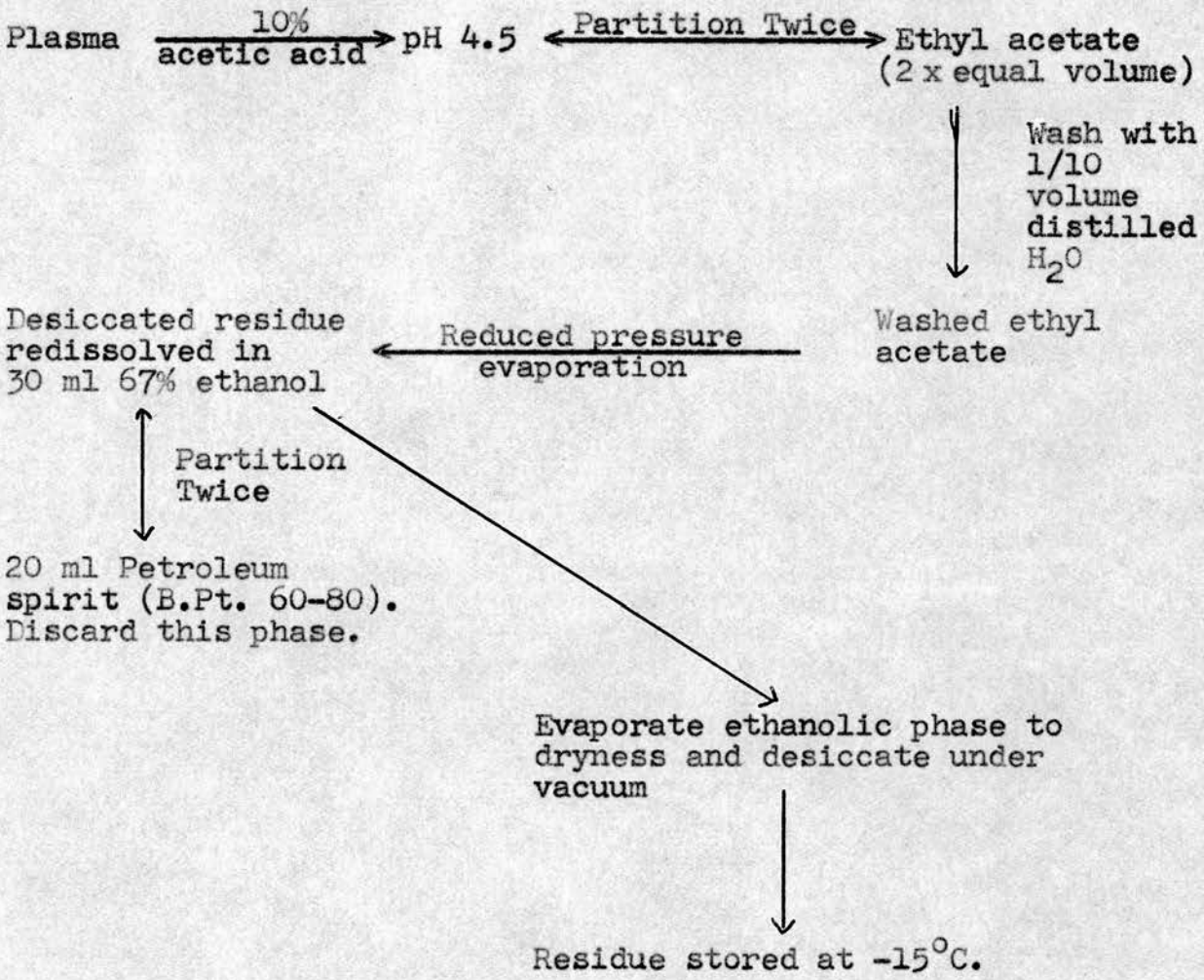
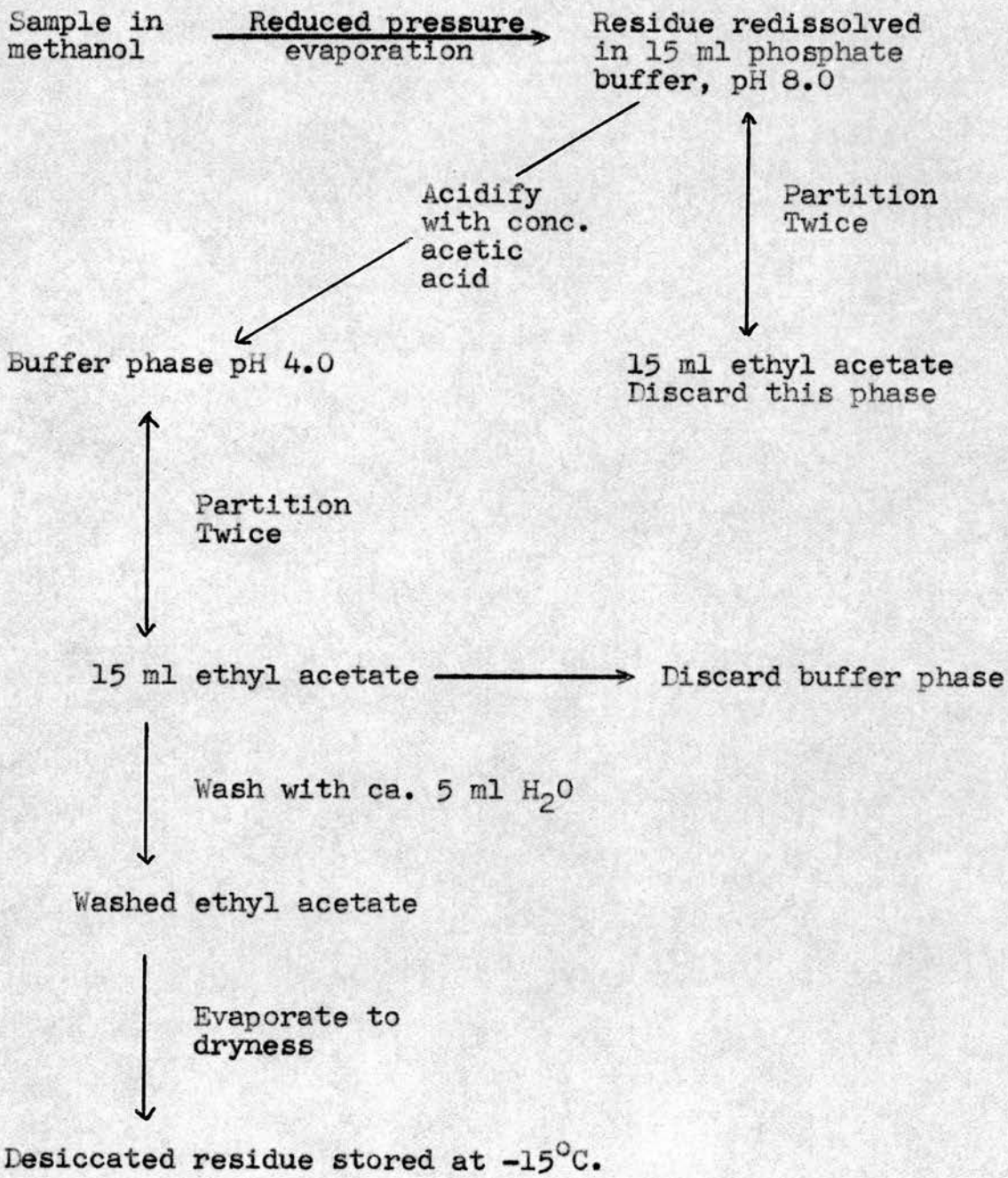


Fig. 1c: Phosphate buffer extraction of PGF samples following thin layer chromatography



petroleum spirit (B.Pt. 60-80). The petroleum spirit phases were discarded, the pear-shaped flask rinsed with a further 15 ml 67% ethanol and the total ethanolic material partitioned with 20 ml petroleum spirit. The petroleum phase was discarded once more and the ethanolic phase evaporated to dryness and desiccated. The residue was stored at -15°C prior to thin layer chromatographic purification and separation of the extract (see Fig. 1b).

Phosphate Buffer Extraction of PGF Samples

following thin layer chromatography, extracts containing PGF were further purified to remove pigmentation so that they would be suitable for mass-spectrometric analysis. The methanolic extract obtained from the chromatography plate was evaporated to dryness and the residue dissolved in 15 ml phosphate buffer, pH 8.0. The buffer was partitioned twice with 15 ml ethyl acetate and this last phase discarded. The buffer phase was taken to pH 4.0 using about 1 drop of concentrated acetic acid and re-extracted twice with 15 ml ethyl acetate. In this instance, the aqueous phase was discarded and the ethyl acetate bulked for washing with ca. 5 ml H_2O to remove acetic acid. The ethyl acetate was then evaporated to dryness and desiccated, after which the residue was suitable for bioassay and for mass spectrometry (see Fig. 1c).

Silica Gel Thin Layer Chromatography

A detailed account of thin layer chromatographic separation of prostaglandins is described by Horton and Main (1967).

Thin layer plates were prepared freshly for each experiment as follows: grooved glass plates, 5 x 20 cm, were coated with a 0.5 mm thick layer of silica gel G (Merck), 45 g silica to 88 ml water. They were then dried and activated by heating at 100°C for 1 hour. The prepared plates were stored in a desiccator until required.

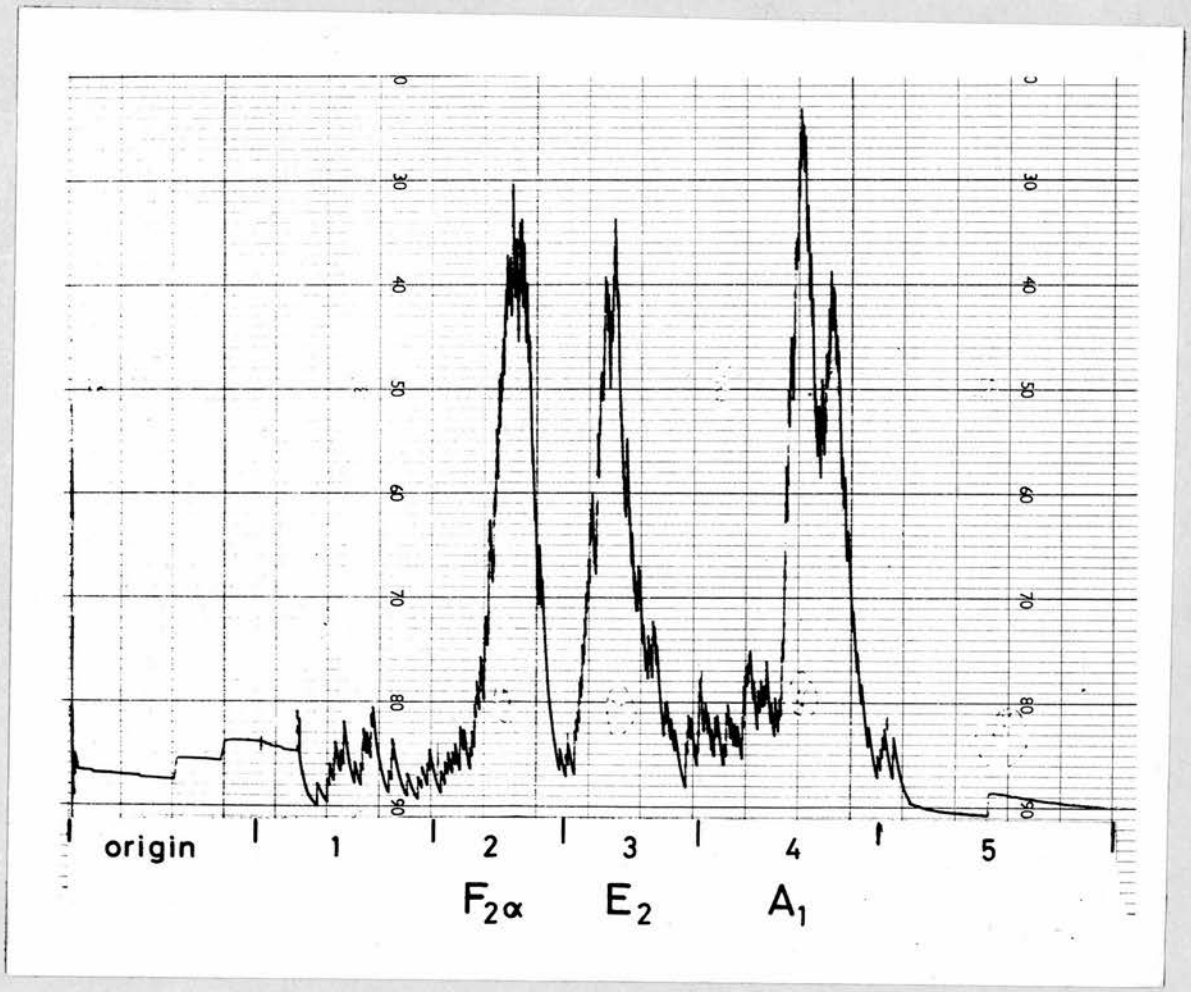
Plasma extracts (containing tritiated marker prostaglandins as forementioned) were dissolved in 0.2 ml methanol and applied as a narrow band to the plates using an Agla micrometer syringe. The flasks were rinsed with a further 2 x 0.1 ml methanol and these washings applied to the band of extract. Following evaporation of the methanol in a stream of cool air, the plates were developed in the AI (modified) solvent system described by Gréen and Samuelsson (1964), which contains toluene:dioxan:acetic acid in a ratio of 20:20:1 respectively. The solvent was allowed to move about threequarters (15 cm) of the way up the plates whereupon the plates were dried in a stream of cool air and scanned with a Panax Radio TLC plate scanner (time constant 10 sec; range 3 cps; paper speed 1 cm/min) to check that the tritiated prostaglandins (and hence endogenous prostaglandins) had traversed from the origin. The area of silica between the origin and the first radioactive peak on each plate was scraped off and retained in a centrifuge tube. This procedure removed much of the pigment which tended to be associated with the origin and prevented its passage further up the plate on subsequent solvent runs.

The plates were then re-run twice in HDII solvent (Davis, 1971), containing toluene:dioxan:acetic acid in a

50:30:1 ratio respectively. This solvent promoted further separation of PGE and PGF with little further movement of PGA. After thorough drying, the plates were scanned once more and the silica marked off into five zones, according to its radioactive content (see Fig. 1d). Each zone was scraped into a centrifuge tube and the silica in each (including the tube containing silica from the origin, removed prior to development in the second solvent system) was washed with 5 ml methanol. The mixture was vortexed and the silica centrifuged down at 1,000 rpm for about 5 min. The methanolic supernatant was removed into a clean centrifuge tube and the silica remaining washed with a further 5 ml methanol. The mixing and centrifugation procedures were repeated and the methanol supernatant removed and bulked with that in the clean tube. This 10 ml methanol was recentrifuged to remove any remaining traces of silica and the supernatant methanol transferred to a pear-shaped flask for evaporation to dryness. Residues from zones 2, 3 and 4 were dissolved in 10 ml methanol and those from the origin, 1 and 5 in 2 ml methanol. Aliquots of 2.0 ml were removed for radioactive counting in 10 ml of a toluene-scintillant fluid containing: toluene, 2.5 ℓ; 2, 5, diphenyloxazole (PPO), 10.65 g; 1, 4-di-(2-(4-methyl-5-phenyloxazolyl)-benzene), 0.275 g. Counting efficiencies for samples in methanol averaged about 45%.

Recovery of tritiated prostaglandins could now be calculated by dividing the total number of disintegrations per minute (dpm) in each particular zone by the total number

Figure 1d: Thin-layer radiochromatographic scan of an extracted plasma sample containing standard tritiated prostaglandins, PGE_2, $PGF_{2\alpha}$ and PGA_1, following development of the plate in AI and HDII solvent systems.



of dpm of appropriate tritiated prostaglandin added e.g. $\frac{\text{Total dpm in zone 2}}{\text{Total dpm } ^3\text{H-PGF}_{2\alpha} \text{ added}} \times 100\%$. Fractions obtained from the origin and zones 1 and 5 were discarded at this stage provided that their radioactive content was less than about 10% of tritiated PGF (origin and zone 1) or tritiated PGA (zone 5) added. In general the content of these zones was considerably less than 10%.

The remaining methanolic fractions from zones 2, 3 and 4 were split into a 40% aliquot for bioassay and a 60% aliquot for combined GLC/MS. All aliquots were evaporated to dryness, desiccated and stored at -15°C.

Biological Assay

Since biological assay was carried out in parallel with combined GLC/MS, assay of each prostaglandin type was confined to one appropriate system.

(a) The rat fundal strip

This preparation was first described by Vane (1957) as suitable for bioassay of 5-hydroxytryptamine. It is, however, also extremely sensitive to prostaglandins of the E and F series. The muscle was cut twice longitudinally to form a zig-zag strip and was then suspended in a 5 ml organ bath containing Tyrode's solution, at 37°C, gassed vigorously with oxygen. PGE and PGF fractions were dissolved in 1 ml distilled water for assay on this preparation. Stocks solution of standard PGE_2 and $PGF_{2\alpha}$ containing 100 ng/ml were prepared. Doses of 5-10 ng were

injected giving a final bath concentration of 1-2 ng/ml. A three minute dose cycle with a drug contact time of 45-60 seconds was used for PGE_2 standards and samples. A slightly longer dose cycle of 5 min with 60-90 second drug contact time was used for $PGF_{2\alpha}$, as it produced marginally slower contractile responses. Contractions elicited by doses of samples were compared quantitatively and qualitatively with those elicited by standard prostaglandin.

(b) Kitten blood pressure assay

As a result of their particularly high sensitivity to the vasodepressor activity of PGA (Horton and Jones, 1969), kittens weighing 1-2 kg, of either sex, were used for assay of fractions containing PGA. The animals were anaesthetised with 40 mg/kg sodium pentobarbitone (Nembutal, Abbott) injected intra-peritoneally and a subsequent maintenance dose of a 6 mg/ml solution was infused intravenously as required. Carotid blood pressure was recorded on a Grass polygraph. Standard solutions of PGA_2 containing 200 and 400 ng/ml were prepared in saline. According to the sensitivity of the preparation, bolus injections (via the femoral vein) of 20-100 ng were used for bracket assay of unknown samples. The 40% aliquots of PGA fractions retained for bioassay were dissolved in 0.4 ml 0.9% saline for bolus injection and were washed in with 0.2 ml saline. A ten minute dose cycle was used for comparison of standard and sample vasodepressor activity.

Combined Gas-Liquid Chromatography/Mass Spectrometry

The gas chromatographic behaviour of prostaglandins is summarised by Ramwell et al. (1968). In order to prevent breakdown of prostaglandins on the GC column, their hydroxyl and ketonic substituents require to be protected by forming stable derivatives and their acid grouping must be esterified. This is effected by (i) methylation of the C : 1 acid group with diazomethane (ii) silylation of the hydroxy and ketonic groups with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) or formation of trifluoroacetate groupings with trifluoroacetic anhydride (TFA).

Fractions remaining after bioassay of PGE, PGF and PGA were pooled with the 60% residues reserved specifically for GLC/MS and taken to dryness. PGE and PGA fractions were converted to the more stable PGB prior to derivatisation to protect them from breakdown on the GC column. This involved treatment with 1 ml 0.1 N potassium hydroxide at room temperature for 1 hour. The PGE samples dehydrate to the corresponding PGA compound under these conditions and PGA isomerises into the more stable PGB conformation. When the reaction was supposed to have gone to completion, the samples were diluted with 15 ml distilled water and the acidity was adjusted to pH 5.0 with conc. acetic acid. They were then partitioned with 2 x 30 ml redistilled ethyl acetate. The ethyl acetate phases were pooled, washed with water, evaporated to dryness and desiccated. The residues of these samples were dissolved in 0.5 ml methanol and transferred to clean Eppindorf micro-tubes. The original flasks were rinsed with further 2 x 0.25 ml methanol and the

washings added to the appropriate Eppindorf tube. The solvent was vaporised off with a fine jet of air and the tubes then desiccated. PGF residues were similarly dissolved in methanol and each sample was divided equally between two Eppindorf tubes for dual derivative formation.

Derivatisation of PGB and PGF

(i) Methylation (Me)

All samples were methylated by addition of fresh diazomethane solution, 0.5 ml, to each Eppindorf tube. The tubes were vortexed and left for 30 min at room temperature, at which stage methylation is complete. The solvent (ether) was vaporised and the samples desiccated.

(ii)a Trimethylsilyl derivatisation of PGB and PGF (Me/TMS)

13 µl of BSTFA (Sigma Chemicals) was added to each sample and the tubes were left at room temperature overnight. (Rapid silylation may be induced by incubation at 60°C for 15 min). 10 µl of each sample in BSTFA was injected onto the gas chromatographic column.

(ii)b Trifluoroacetate derivatisation of PGF (Me/TFA)

Trifluoroacetic anhydride (TFA, Koch-light Labs.) was added by Pasteur pipette (about 0.5 ml) to the duplicate methylated PGF samples. The Eppindorf tubes were closed and left at room temperature for 2 hours. The TFA was then removed by evaporation under water vacuum in a desiccator. Samples were redissolved in 13 µl hexane for injection (10 µl) onto the GC column.

Preparation of Authentic Prostaglandin Derivatives

The Me/TMS derivatives of PGB_2 and $PGF_{2\alpha}$ (1 µg of each) were prepared in 25 µl BSTFA. Similarly, 1 µg of Me/TFA $PGF_{2\alpha}$ was prepared in 25 µl hexane.

Standards were injected on to the GC column in 5 or 10 µl aliquots and retention times noted for comparison with the unknown samples.

GC/MS Analysis

Analysis was performed using an LKB 9000 gas chromatograph-mass spectrometer. The column (3.0 m x 1.5 mm i.d.) was packed with 3% OVI on a Supelcoport pretreated with dimethylchlorosilane. The column temperature was 190-200°C and the flow rate of the carrier gas, helium, was 30 ml/min.

Authentic prostaglandin derivatives were injected on to the column as described and a mass spectrum of each standard was taken at the peak of its chromatogram. Extract derivatives were then injected on to the column and mass spectra were taken at retention times corresponding to those obtained with authentic prostaglandin derivatives (see Results, Figs. 1j, 1k and 1ℓ).

(b) A Critical Appraisal of Bioassay of Prostaglandins by the Blood Superfusion Technique in Rabbits

Introduction

Prior to the development of radioimmunoassay and multiple ion detection mass spectrometry in the laboratory, the techniques available for prostaglandin assay involved principally those extensive procedures of extraction,

purification, bioassay and combined GLC/MS previously described in this section. Thus the complete purification and identification of prostaglandins in a series of blood samples took several weeks. In the hope of obviating the need for such lengthy procedures, which inevitably reduce productivity, it was decided to investigate the use of the superfusion bioassay technique first described by Gaddum (1953). This technique has recently been developed by Vane (1969) for the assay of a number of blood-borne substances. Blood (or perfusion fluid) is passed over the surfaces of a series of smooth muscle preparations which have been selected for their sensitivity to the endogenous substance(s) being measured. The presence of substances is recognised by appropriate contractions or relaxations of the tissues and quantitation involves a comparison of these effects with those produced by standard solutions of the substance in question. In addition to tissue selectivity, additional specificity may be attained by the use of specific blocking drugs to eliminate effects produced by other endogenous substances. The advantage of this technique is that it affords on-the-spot monitoring of endogenous substances. Blood samples may be collected as appropriate for more refined analysis and absolute identification. It was the intention in present experiments to utilise this method as a means of discovering which doses of noradrenaline, if any, were effective in stimulating release of prostaglandins from the rabbit kidney. Control blood samples could be collected as usual and test samples

when there was an indication of altered biological activity.

Methods

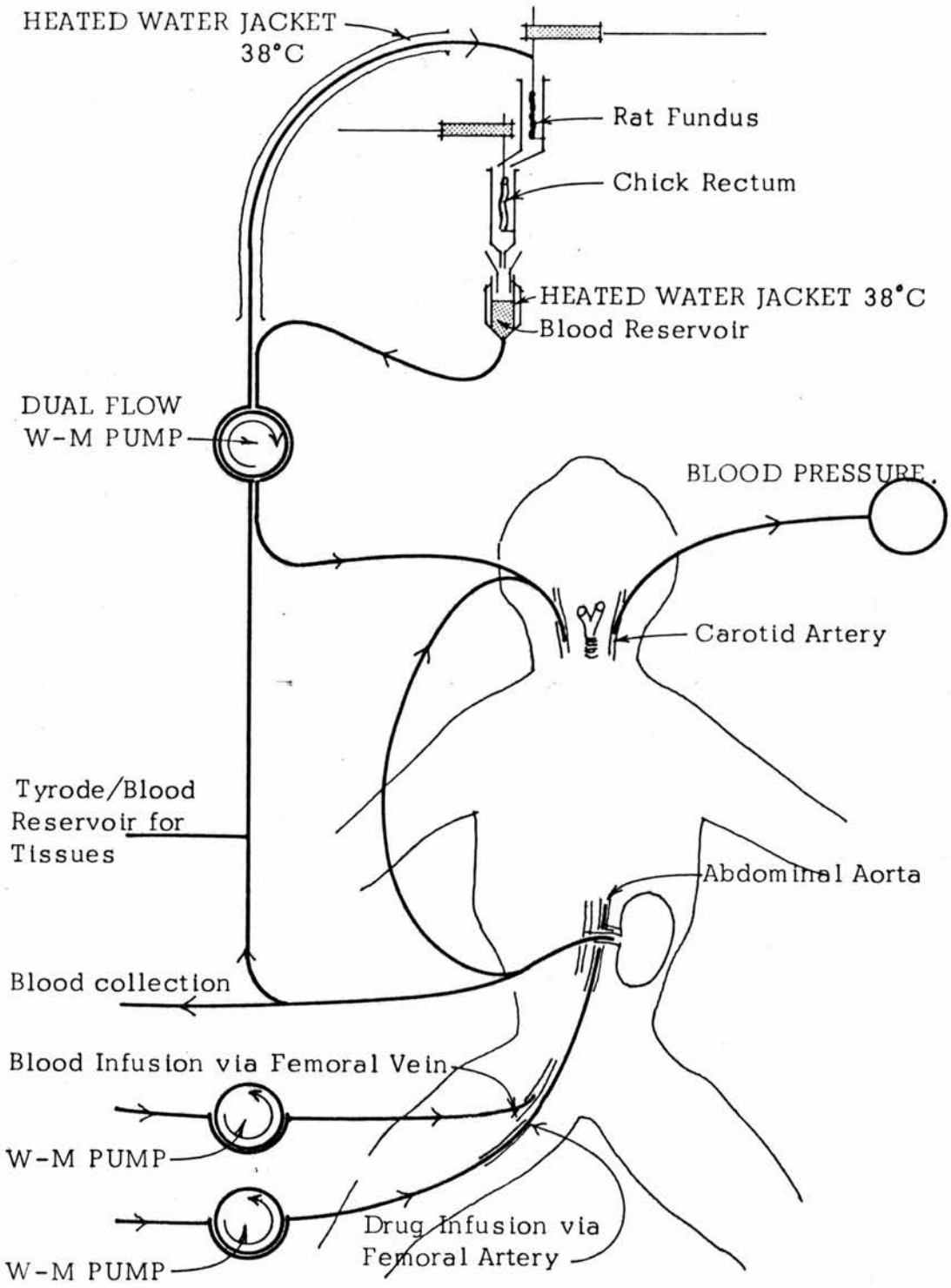
The tissues selected for their sensitivity to prostaglandins were the rat fundal strip and the chick rectum. The specificity of these tissues was increased by soaking them in appropriate antagonist solutions in Tyrode for about an hour prior to setting them up. Adrenaline relaxes both tissues whilst noradrenaline relaxes the rat stomach with little effect on the chick rectum. In addition, 5-hydroxytryptamine (5-HT) contracts the rat stomach, having little effect on the chick rectum. Prostaglandins contract both tissues. It was decided, therefore, to soak the tissues in a mixture of the 5-HT antagonist, bromlysergicdiethylamide (5×10^{-7}M) and the β-blocker, propranolol (5×10^{-6}M). Vane (1969) provides a comprehensive list of other hormones which do not affect these tissues at normal blood concentrations. The list includes angiotensins I and II, vasopressin, bradykinin, ATP, corticoids, gastrin, histamine, kallikrein, oxytocin, renin and SRS-A.

Subsequent to antagonist treatment, the preparations were set up in series with the fundus (tension 2 g) above the rectum (tension 5 g). They were cascaded initially with Tyrode's solution at 37°C, gassed with O_2. A flow rate of 2.4 ml/min was used. The tissues contracted to a 5 ng/ml standard solution of PGE_2 in Tyrode, responses being recorded isometrically from Statham (P23 DC) pressure transducer onto a Devices recorder.

Eight experimental rabbits weighing 2.0 to 2.5 kg were set up in a manner similar to that described under "Experimental Procedures" and, following dissection, were injected intravenously with heparin (1 i.u./g body wt.). When the isolated tissues had stabilised in Tyrode's solution, the latter was replaced by renal venous blood from the experimental animal, similarly cascading at a rate of 2.4 ml/min over the tissues (Fig. 1e). Blood from a donor rabbit (usually a litter-mate to lessen the risk of incompatibility) was infused into the experimental animal at the onset of cascade to replace the blood in the circuit. The renal venous outflow in excess of that used to cascade the tissues was returned to the animal via its external jugular vein. It could also be collected through a side-arm for further analysis. Blood from the assay tissues was collected in a heated polythene reservoir maintained at 38°C, and was returned to the animal via the external jugular vein at the same rate (using a dual-flow Watson-Marlow pump) as that blood being removed for cascade.

A fine cannula (P50) was inserted into the right femoral artery and pushed up into the abdominal aorta such that its tip lay just rostral to the left renal artery. After equilibration of the isolated tissues in renal venous blood, noradrenaline, NA (250 ng/kg/min) was infused through the aortic cannula. In two experiments, carried out in collaboration with Dr. H.A. Davis, antidiuretic hormone (ADH) was infused in place of noradrenaline in doses ranging from 100 μ units to 20 m units/kg/min.

Figure 1e: Blood superfusion technique.



Results

In two experiments, NA was infused into the abdominal aorta at a rate of 250 ng/kg/min. This rather high dose was chosen as a result of the apparently negligible effect of 100 ng/kg/min on renal prostaglandin output in a previous experiment where blood samples were collected for bioassay/GLC/MS analyses. A dose of 250 ng/kg/min has also been employed in the dog using the superfusion assay technique to measure prostaglandins (McGiff et al., 1972). When NA was infused into the rabbits as above, an increase in systemic blood pressure was observed, followed by an eventual large decrease in renal blood flow. The decrease in blood flow was sufficiently great to render the cascade system inoperable, thus defeating its purpose. The tissues were switched temporarily to a cascade of arterial blood from an emergency reservoir (obtained from a donor animal) so that a further infusion of a lower dose of NA might be attempted. Renal blood flow did not return to the pre-infusion level and the condition of the animals gradually deteriorated until they were hypotensive and soon died.

A further four experimental rabbits were set up to test the effect of lower doses of NA on renal blood flow and prostaglandin output. All four animals died soon after being set up with their renal venous blood superfusing the isolated tissues. Death in each case occurred prior to infusion of noradrenaline.

In the two experiments in which ADH was used, a rise in systemic blood pressure was observed when doses from

6 mu/kg/min were infused intra-aortically. Changes in the tone of the isolated preparations were observed, as indicated below.

Changes in blood pressure and tissue tone observed during infusion of ADH

	Dose of ADH per kg min^{-1}	B.P. Sys/Dias	Rectal Tone	Fundic Tone
Exp. 1	100 µu	No change	No change	No change
	200 "	" "	" "	" "
	400 "	" "	" "	" "
	800 "	" "	" "	" "
	1.6 mu	" "	" "	" "
	3.2 "	" "	Rise	" "
	6.0 "	68/33 to 72/39	Fall	Fall then rise
Exp. 2	6 mu	62/29 to 65/36	Rise	Slight fall
	20 "	65/33 to 80/60	Rise	Fall then rise
	10 "	80/47 to 94/68	Rise	Fall then rise

In Experiment 1, the responses of the isolated tissues during ADH were inconsistent, making interpretation of the tone changes in terms of prostaglandin concentration impossible. Following infusion of ADH at 6 mu/kg/min, the systemic blood pressure rose initially but then fell until the animal was severely hypotensive and died.

In Experiment 2, the responses of the isolated tissues during infusion of higher doses of ADH, 6-20 mu/kg/min, were consistent and do indicate that prostaglandin concentration in the renal venous blood had probably increased. The condition of this animal did not deteriorate so rapidly.

Conclusion

Despite the precautionary measures taken to supplement the experimental animals' blood loss and to maintain oxygenation and warming of the blood returning to the animals from cascade, it was generally found that rabbits could not withstand the conditions of the cascade experiment for long periods of time. Infusion of noradrenaline and ADH, which may have resulted in increased prostaglandin concentrations in the renal venous blood, may, in part, have been responsible for the severe hypotensive states which followed when the blood from cascade was returned to the animals. However, in four out of eight experiments, the animals died prior to any drug infusion. It is possible that active substances (probably prostaglandins) were released from the isolated tissues themselves and were then infused into the animal in the blood from cascade. On reflection, it would have been preferable to soak the isolated tissues in indomethacin to block endogenous prostaglandin synthesis, as well as in the selected antagonists.

The survival rate of the animals would almost certainly have been improved if the blood from cascade had not been re-infused. This would undoubtedly have

led to problems, however, in blood-transfusing the animal with compatible blood for a sufficient period of time. In addition, it would require a large number of donor rabbits.

In conclusion, the blood superfusion technique, although successfully used by others in dogs, proved unsuccessful in present rabbit experiments. The renal outflow from the dog kidney is sufficiently large, even during marked vasoconstriction, to permit continued superfusion of isolated tissues (McGiff et al., 1972). This is evidently not so in the rabbit. Neither does the rabbit respond well to re-infusion of cascade blood.

In view of these difficulties, attempts to measure prostaglandin output in blood from the rabbit kidney using the cascade method were abandoned.

(c) Radioimmunoassay

The development over the past few years of a radioimmunoassay for prostaglandins has permitted an increase of about one hundred fold in sensitivity of detection of these substances. Whereas bioassay is capable of detecting amounts of prostaglandin in the nanogram range, radioimmunoassay is capable of detecting tens of picograms. The success

in raising antibodies to $PGF_{2\alpha}$ and PGB_2 in the Edinburgh laboratory led to the development of sensitive assays for these prostaglandins. A combination of sensitivity, specificity and facility for assay of large numbers of samples constituted good reason for adaptation and use of radioimmunoassay in preference to the traditional bioassay for measurement of prostaglandin levels in renal venous samples.

The method is dependent upon the immunogenic properties of the substance being measured. The prostaglandins, which are of relatively low molecular weight and are not in themselves immunogenic, must be conjugated to a macromolecule such as bovine serum albumin. Antisera raised in this way contain a mixed population of antibodies but only those which are directed towards the prostaglandin (which is the hapten) will be involved in reactions with the free hapten. The reaction of these antibodies may be followed by using a radioactively-labelled hapten (tritiated prostaglandin). At the concentration of antigen used in such assays, the antibody-antigen complex (Ab-Ag complex) remains in solution. Thus, in order to follow the reaction of the labelled antigen with the antibody, it is necessary to separate Ab-bound labelled antigen from free labelled antigen. In the present system this was effected by precipitation of the bound antigen using a second antibody which was raised to the γ-globulin of the original antibody, in another species, the donkey. The second antibody will be referred to as donkey anti-rabbit serum. This method is known as the double antibody method and it relies upon the finding that the

antigenic site(s) of an antibody are separate from its antibody sites. An antibody molecule may form a complex with its antigen and then itself be complexed to a second antibody. In radioimmunoassay, however, the primary Ab-Ag complex is too dilute to be precipitated and requires the presence of non-immune serum belonging to the same species as the first antibody (referred to later as normal rabbit serum). If this is added and is then followed by the second antibody, a sizeable lattice can be built up and the whole, including labelled antigen bound to the first antibody, is precipitated. The percentage bound is determined by measuring the radioactivity present in the supernatant after centrifugation and subtracting this from the initial amount of labelled antigen added. (Precipitation is generally complete after 16 hours at 4°C and this was found to be the case in the present system.) As the amount of cold antigen in the medium increases, the greater is its competition with the labelled antigen for binding sites on the antibody. Hence, the percentage of bound labelled antigen decreases. Reductions of less than 10% below the binding of zero standards (containing all reagents except cold prostaglandin) are not accepted as accurate, however, as this part of the dose/response curve is inherently imprecise. Factors which may affect the specificity of the assay include tracer homogeneity and background medium interference e.g. from plasma or from solvent residues resulting from extraction of samples. These effects may be additive and will be relatively most significant where the concentration of the antigen approaches

the detection limit of the system.

For further information on radioimmunoassay, the reader is referred to the excellent account by Hunter (1973). A detailed description of all procedures employed in this section is presented by Dighe, Emslie, Henderson, Simon and Rutherford (1975).

Methods

(a) Development of antisera

In the Edinburgh laboratory, antisera to PGB_2 and to $PGF_{2\alpha}$ were raised in rabbits. This was effected by immunising the rabbits with appropriate prostaglandin-bovine serum albumin conjugates. Of the various antisera obtained, those exhibiting a combination of high specificity (i.e. low cross-reactivity with prostaglandins other than that to which they were raised) and high "titre" (i.e. high PG-binding capacity per unit volume) were selected for routine assay of prostaglandins. The "titre" of an antiserum was obtained by constructing a dilution curve of % binding of tracer-PG by antiserum VS. dilution of antiserum. The "titre" was read as that dilution which bound 60% of the tracer-PG in the absence of non-radioactive standard prostaglandins. This value was chosen since from about 70% binding downwards to about 50%, a straight line is obtained when the abscissa is plotted logarithmically.

Antisera titres

The antisera utilised for rabbit renal venous plasma estimations of $PGF_{2\alpha}$ and PGB_2 had the following titres:

$PGF_{2\alpha}$ antiserum	1 in 11,200 dilution to bind 150 pg ^{3}H-$PGF_{2\alpha}$ (35.6 ng/μCi)
PGB_2 antiserum	1 in 24,800 dilution to bind 20 pg ^{3}H-PGB_2 (2.5 ng/μCi)

Specificity of Antisera

Table 1a lists the percentage cross-reactivity of $PGF_{2\alpha}$ antiserum (using two differing tracer specific activities) and PGB_2 antiserum with other prostaglandins. The values indicate a high cross-reactivity of the antisera with the corresponding 1-series prostaglandins, $PGF_{1\alpha}$ and PGB_1, but otherwise show these antisera to be reasonably specific.

(b) Reagents used in radioimmunoassay

A diluent of the following composition was used for diluting all reagents used in the assay: 0.05 M tris-hydrochloride buffer (pH 8), sodium azide (0.1 g/l) and gelatine (1 g/l).

Tritiated prostaglandin $F_{2\alpha}$ (35.6 ng/μCi; New England Nuclear Company), and ^{3}H-PGB_2 (2.5 ng/μCi; Amersham) were dissolved in diluent to give the following concentrations:

Tracer		Concentration	Amount per 50 μl
^{3}H-$PGF_{2\alpha}$	35.6 ng/μCi	3 ng/ml	150 pg
^{3}H-PGB_2	2.5 ng/μCi	0.4 ng/ml	20 pg

PG-antisera were diluted with diluent so that when 50 μl was added to the reaction tube (in which the reaction volume was 1.2 mls) the final dilution of the antiserum was

Table Ia

Cross-reactivity of antisera (%)

PG tested	Antisera + titres for given tracer-PG	
	$PGF_{2\alpha}$ 1 in 11,200 150 pg ^{3}H-$PGF_{2\alpha}$ (35.6 ng/μCi)	PGB_2 1 in 24,800 20 pg ^{3}H-PGB_2 (2.5 ng/μCi)
E_2	0.7	7.0
E_1	0.5	2.0
A_2	0.003	10.0
A_1		6.0
B_2	0.1	
B_1		18.0
$F_{2\alpha}$		0.004
$F_{2\beta}$	0.3	
$F_{1\alpha}$	73.0	
15 OXO $F_{2\alpha}$	0.2	
dihydro B_1		2.0

equal to its "titre". The second antibody, donkey anti-rabbit serum (DARS) (Wellcome Reagents, Ltd) was used as a 3:7 dilution in diluent. Normal rabbit serum (NRS), obtained from non-immunised donors, was used as a 1:53 dilution in diluent. (The methods for determining the concentration of NRS and DARS required for radioimmunoassay have been described by Hunter (1973)).

A scintillation fluid with the following composition was used for radioactive counting: naphthalene 75 g; PPO 7 g; DMPOPOP 0.6 g; 2-ethoxyethanol, 600 ml; toluene 1 litre. A volume of 13 ml scintillant absorbed 0.6 ml of an aqueous solution, counting efficiency being approximately 30%.

(c) Preparation of standard curves

Standard curves were set up for each antiserum covering various ranges of cold standard prostaglandin concentration (see Table 1b and Fig. 1f). A standard curve was included as a control in every radioimmunoassay of unknown samples. Each curve consisted of duplicate tubes containing a range of prostaglandin concentrations contained in a volume of 1 ml diluent. In addition, zero standards (to check binding capacity of the antiserum), blanks (to check for non-specific binding of tracer to any of the other reagents) and counting standards (to check number of counts added in 50 µl of tracer) were set up in quadruplicate (Table 1c), each tube containing 1 ml diluent in place of standard prostaglandin solution.

Tracer solution (50 µl) was added to each tube and the solutions were mixed and equilibrated for about 10 min.

Table Ib

Amounts (ng) of PGB_2 and $PGF_{2\alpha}$ used to construct standard curves.

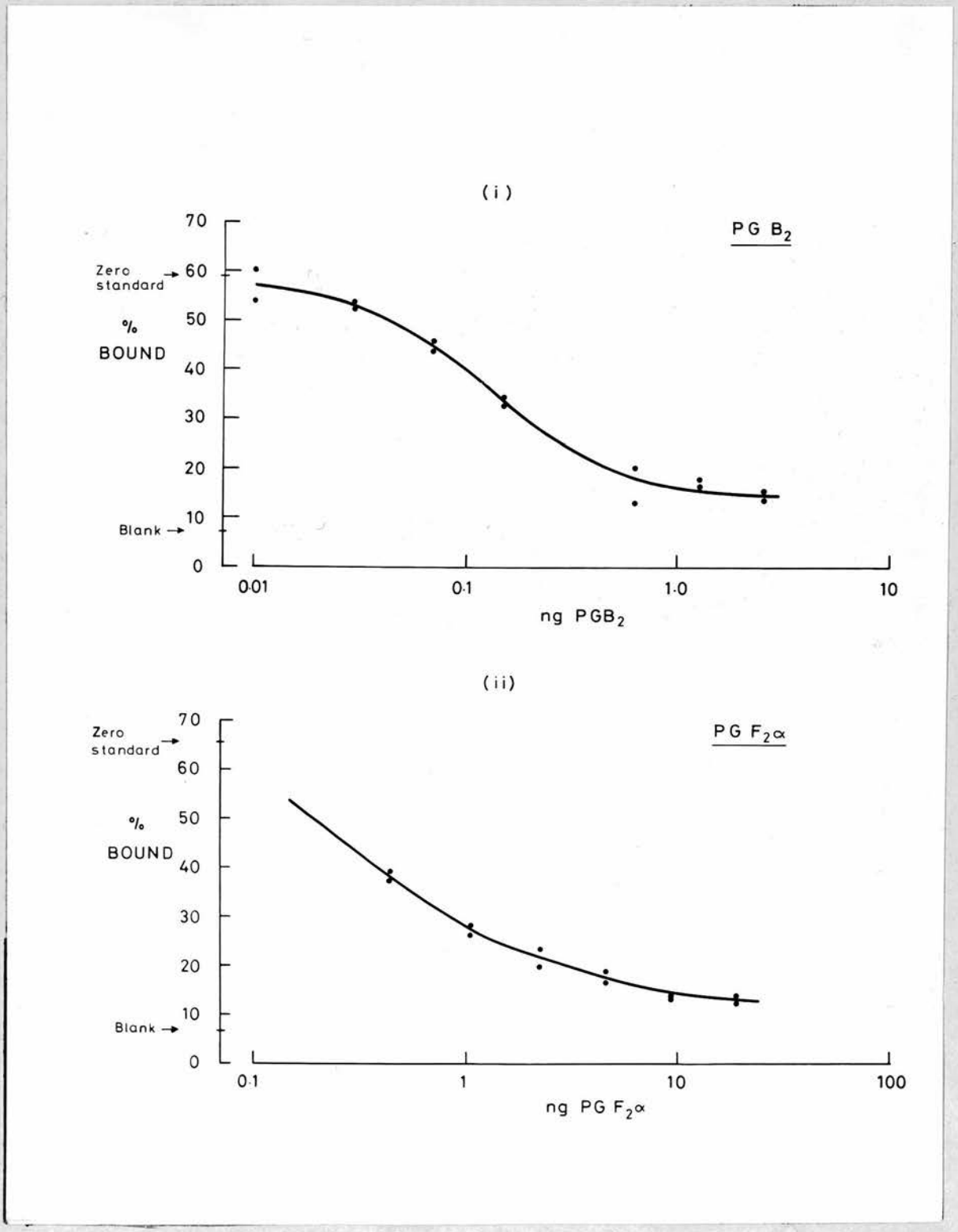
$PGF_{2\alpha}$ (35.6 ng/μCi)	PGB_2 (2.5 ng/μCi)
0.15	0.01
0.45	0.02
1.05	0.03
2.25	0.07
4.65	0.15
9.45	0.31
19.05	0.63
	1.27
	2.55

Table Ic

Reagents

Tubes	Cold PG	Diluent	Tracer	Anti-serum	NRS	DARS
Standard curve	1 ml	-	50 μl	50 μl	50 μl	50 μl
Blanks	-	1 ml	50 μl	-	50 μl	50 μl
Zero standards	-	1 ml	50 μl	50 μl	50 μl	50 μl
Counting standards	-	1 ml	50 μl	-	-	-

Figure 1f: Standard curves obtained by radioimmunoassay of standard prostaglandins B_2 and $F_{2\alpha}$. The means of four blank samples are represented for each curve. Dots represent duplicate binding values obtained for each prostaglandin amount. The abscissa is plotted on a logarithmic scale.



50 µl diluted antiserum (as described in (b)) was then added to all tubes excepting blanks and counting standards and, after mixing, the samples were incubated at room temperature (18-20°C) for one hour. After incubation, NRS (50 µl) was added, followed, after mixing, by DARS (50 µl) to all tubes excepting counting standards. The tubes were mixed once more before incubating for 16 hours at 4°C. After incubation, the samples were centrifuged at 4°C, 1200 g for 45 minutes. 0.6 ml aliquots of supernatant were removed from each tube and added to counting vials containing 13 ml scintillant. The vials were monitored for radioactivity in a Nuclear Chicago liquid scintillation counter, using the external channels/ratio method and counting each vial for 4 or 10 min.

(d) Preparation of plasma samples for radioimmunoassay

As in experiments employing bioassay and mass spectrometry, it was desirable to measure PGE_2, $PGF_{2\alpha}$ and A, C or B-like prostaglandins in rabbit renal venous plasma. The only antibodies available were those to PGB_2 and $PGF_{2\alpha}$. Consequently, PGE and PGA or PGC had to be converted to PGB for assay. A series of control experiments was carried out to study the effects of plasma extraction and of chemical conversion procedures on radioimmunoassay.

Conversion of PGE to PGB

PGE was converted to PGB by dehydration treatment with methanolic KOH. This treatment also resulted in conversion of PGA and PGC to PGB, but since there was also

a separate assay of these prostaglandins following incubation, their concentration only required to be subtracted from the total PGB_2 assayed, thus leaving the PGB_2 which resulted from PGE_2 dehydration.

A series of control experiments was carried out to determine optimum conditions for the conversion of plasma PGE_2 to PGB_2. Rabbit aortic blood was used as a background medium for conversion of standard PGE_2 solutions to PGB_2 as it contains very little, if any, endogenous prostaglandin. Two procedures of dehydration were attempted at varying temperature, time and KOH concentration. (Fig. 1g):
(i) Incubation of plasma with 1 ml methanolic KOH, followed by acidification and extraction into ethyl acetate. Ethyl acetate evaporated under reduced pressure, the residue desiccated and made up in diluent for radioimmunoassay.
(ii) Acidification and extraction of plasma into ethyl acetate, followed by reduced pressure evaporation and desiccation of the ethyl acetate residue. Incubation of residue with 1 ml methanolic KOH, addition of 15 ml water and reacidification of sample. Re-extraction with ethyl acetate, vacuum evaporation and desiccation and addition of diluent for radioimmunoassay. The results are summarised in Table 1d.

It was concluded that the best conditions of those investigated for conversion of PGE_2 to PGB_2 were incubation of plasma with 1 ml 1N KOH in methanol at $45^{\circ}C$ for one hour. These conditions were subsequently employed for renal venous sample analyses.

Figure 1g: Dehydration and extraction procedures for the radioimmunoassay of PGE_2 as PGB_2

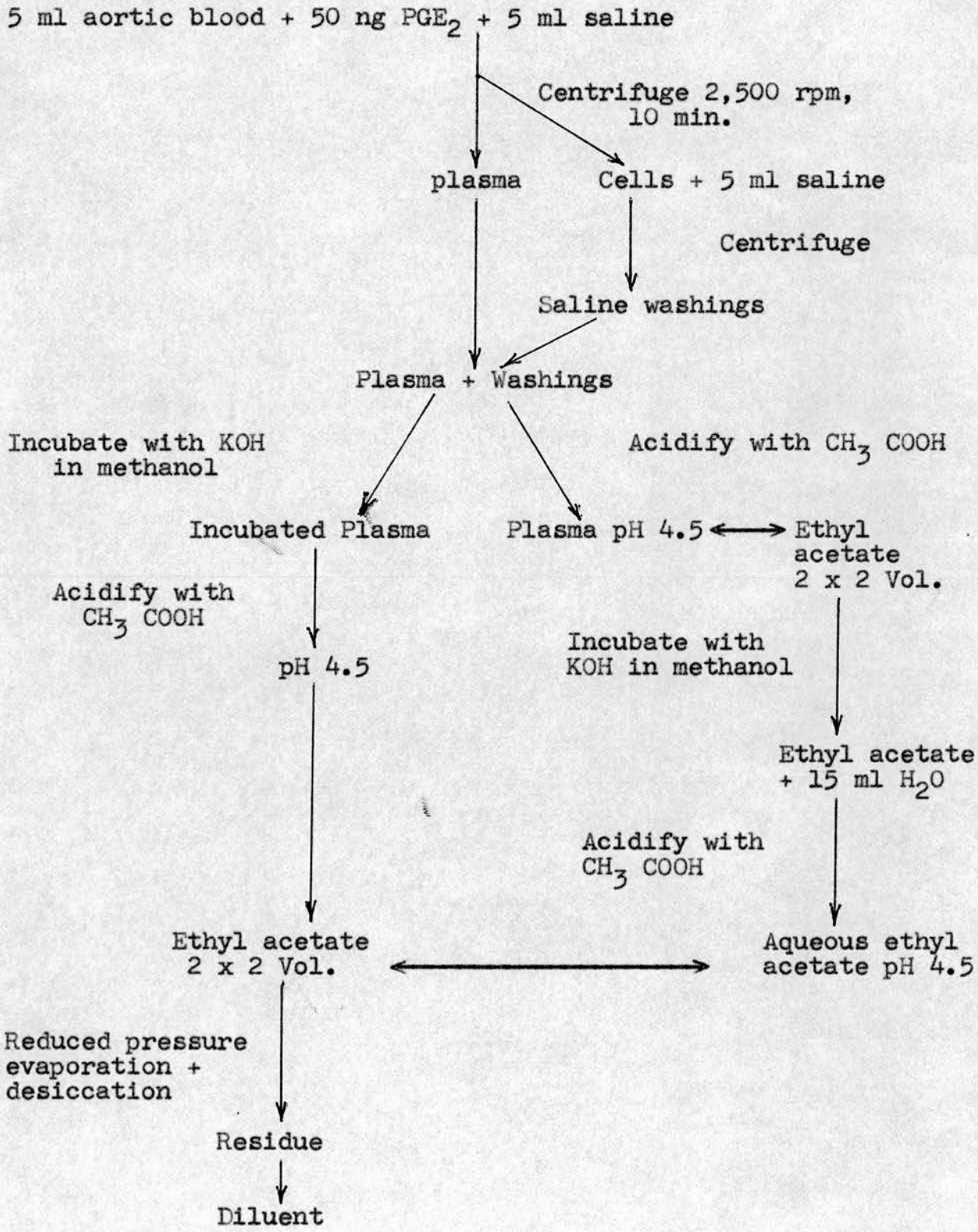


Table 1d

Conversion by dehydration of PGE_2 to PGB_2

Values represent assayed concentrations of PGB_2 (ng/ml). Where the number of observations exceeds two, mean values ± S.E.M. are quoted and the number of observations is indicated within brackets.

KoH concn.	Temperature (°C)	Incubation Time (hours)	Aortic blood + 10 ng/ml PGE_2		Aortic blood (no PG added)		Diluent + 10 ng/ml PGE_2
			Plasma	Extracted Plasma	Plasma	Extracted Plasma	
0.1 N	45	0.5	) 2.8) 3.1				
0.1 N	45	1.0	3.8 ± 0.19 (8)		0.5	0.25	4.6
0.1 N	45	2.0	4.5 ± 0.14 (6)		0.4		5.2
1.0 N	45	1.0	) 5.6) 6.0	3.0	0.4	0.6	
0.1 N	70	0.5	) 3.3) 3.6	) 2.2) 2.4			
0.1 N	70	1.0	3.2 ± 0.18 (4)				
1.0 N	70	0.5		) 2.2) 2.4			
1.0 N	70	1.0	) 4.6) 4.8	) 1.4) 1.6		0.24	3.5

Conversion of PGA or PGC to PGB

Rabbit plasma is known to contain an enzyme, prostaglandin isomerase, which rapidly isomerises PGA to PGC. The latter is very unstable and converts quickly to the more stable isomer, PGB (Jones, 1970, 1972). It was assumed, therefore, that any PGA or PGC-like compounds released into renal venous blood would be rapidly converted to PGB prior to assay. To ensure complete isomerisation, however, renal venous plasma was reheated to 38°C for two hours to reactivate the isomerase. (This procedure was carried out using standard PGA_2 in Aortic blood by Dr. H.A. Davis, results in press).

Following incubation, the renal venous plasma was acidified and extracted into ethyl acetate (as described for PGE_2), the ethyl acetate was evaporated under reduced pressure and the residue vacuum-desiccated. The residue was taken up in diluent for radioimmunoassay.

Extraction of $PGF_{2\alpha}$ from plasma

A series of control extractions was performed with aortic blood as a background medium for assay of standard $PGF_{2\alpha}$ solution. $PGF_{2\alpha}$ was added to blood to obtain concentrations of 1 and 10 ng per ml blood. The plasma was removed, acidified and extracted with ethyl acetate. The residue remaining after evaporation was dissolved in diluent for radioimmunoassay (cf. procedure with that for PGE and PGB).

The mean results are summarised in the table below:

Table Ie

Control radioimmunoassay of $PGF_{2\alpha}$. Figures in brackets indicate number of experiments.

Background medium	$PGF_{2\alpha}$ added per ml	Assay results ng/ml
Blood	None	0.8 1.0
Blood	1 ng	2.0 2.3
Blood	10 ng	10.4 10.6 10.3 10.5
Saline	10 ng	10.5 9.7

It was concluded that the standard prostaglandin extraction procedure did not interfere with the radioimmunoassay of $PGF_{2\alpha}$ and, therefore, might be used for measurement of $PGF_{2\alpha}$ in renal venous samples.

(d) Multiple Ion Detection Mass Spectrometry

A multiple ion detection (MID) system using a Finnigan 3000D quadruple mass spectrometer was developed for the sensitive quantitative assay of prostaglandins E_2 and $F_{2\alpha}$, either separately or in parallel (Hensby and Naylor, 1974). This procedure offers three major advantages over radioimmunoassay:

(1) Mass spectrometry provides absolute identification.

(2) PGE may be assayed without conversion to PGB.

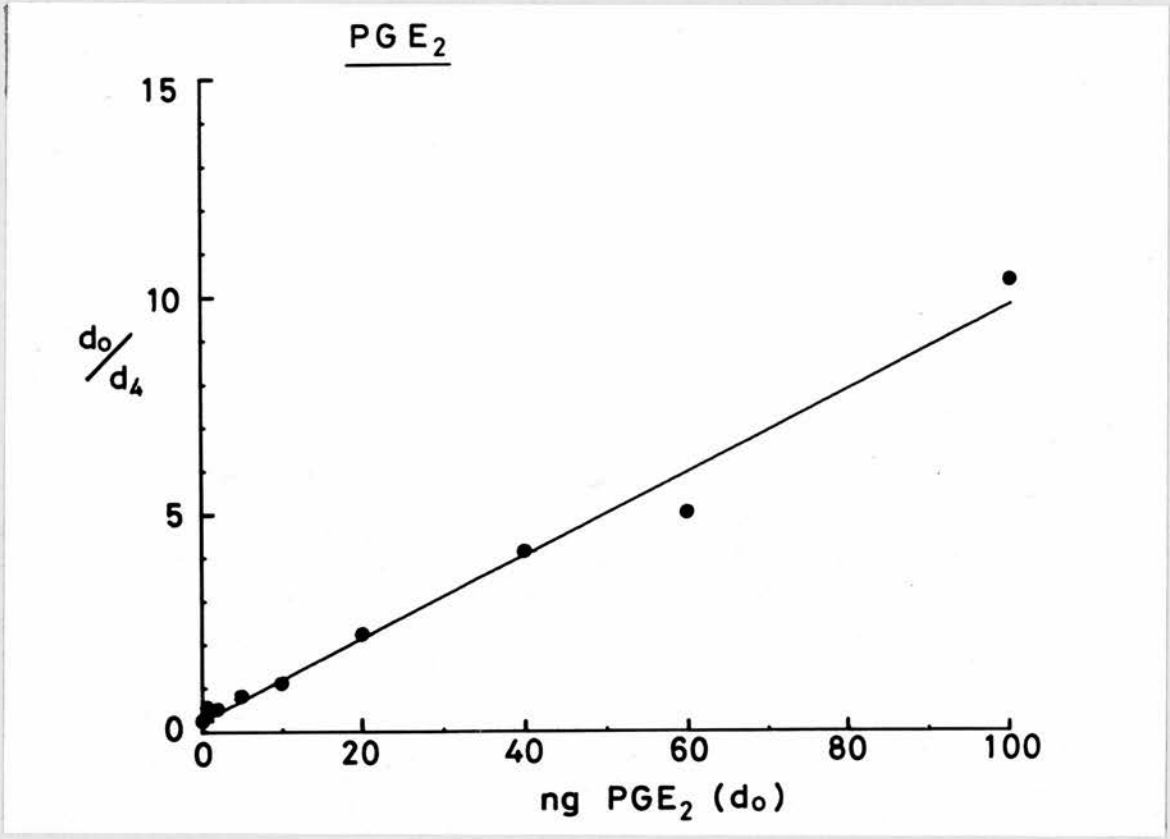
(3) PGE and PGF may be assayed in parallel.

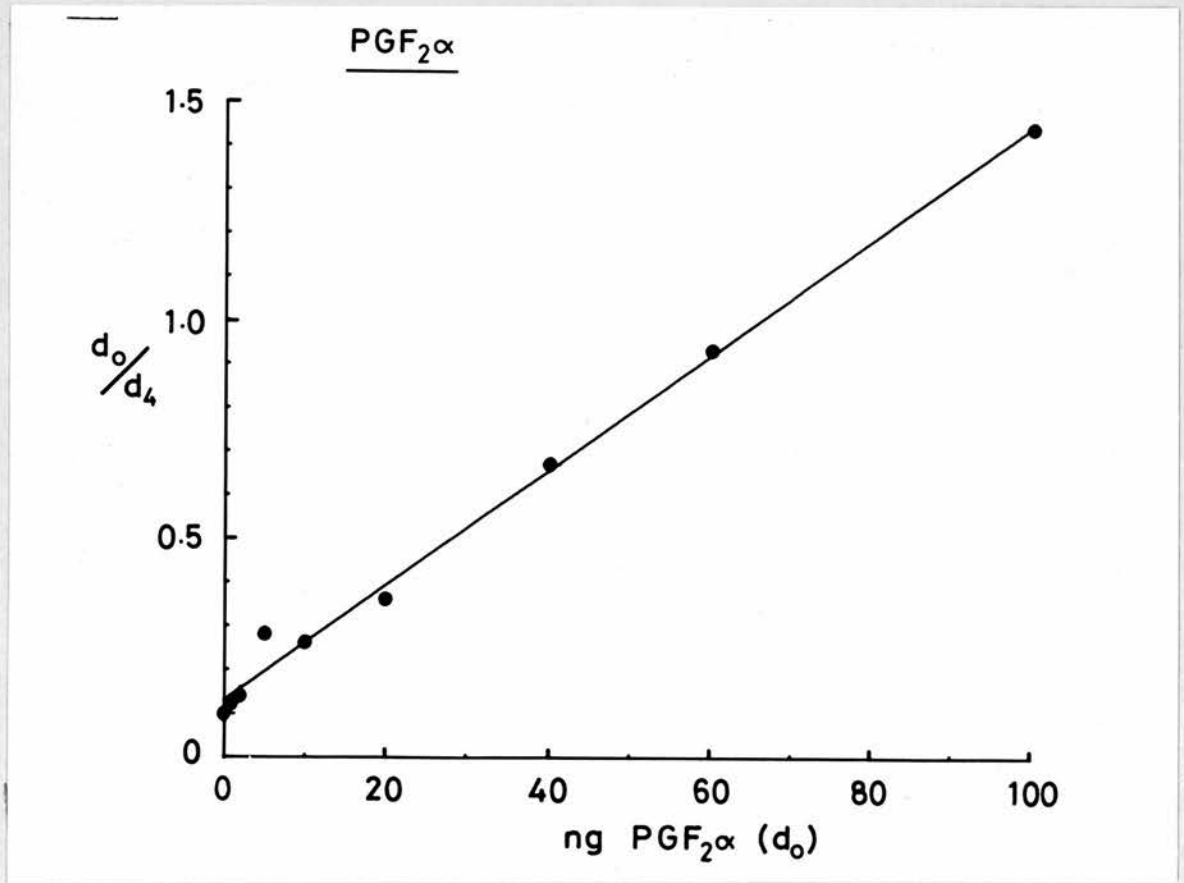
The method is based on the reversed isotope dilution technique described by Samuelsson, Hamberg and Sweeley (1970). A known amount (in this case, 1 μg) of the corresponding deuterium isotope (d_4) is added to the unknown sample of prostaglandin and the two, when derivatised, co-chromatograph. The excess of the deuterated derivative acts as a carrier for the sample prostaglandin both through the column and the separator. This method has the added advantage of quantitation using the deuterated prostaglandin as an internal standard. By comparing the intensity of the chromatographic peaks obtained from the deuterated prostaglandin and the sample prostaglandin, a peak ratio may be calculated. A calibration curve is obtained by plotting the ratio of protium (d_0) to deuterium (d_4) peak heights over a range of protium amounts (in this case, 1-100 ng), deuterium being kept constant (1 μg).

Preparation of calibration curves

For each set of samples analysed by MID, it was necessary to construct a calibration curve (Fig. 1h). Each standard contained a mixture of PGE_2, $PGF_{2\alpha}$, d_4 PGE_2 and d_4 $PGF_{2\alpha}$. A plot of peak ratios (protium/deuterium) versus amount of protium produced a straight line between 0 ng and 100 ng protium (and up to 1000 ng protium; Hensby, personal communication). Hence, by calculating the peak ratio in an unknown sample, it is possible to read off the protium content from the calibration curve.

Figure 1h: Calibration curves for assay of PGE_2 and $PGF_{2\alpha}$ by multiple ion detection. Ordinate = ratio of protium/deuterium ($\frac{d_o}{d_4}$). Abscissa = amount of protium (d_o).





Control analyses of "mock" biological samples

The samples analysed by MID consisted of Dextran-Tyrode perfusate obtained from the isolated kidney preparation. It was necessary to carry out control extraction and mass spectrometric procedures on standard solutions of prostaglandins in Dextran-Tyrode solution to check that this medium did not interfere with prostaglandin extraction and analysis. A small amount of tracer prostaglandin was mixed with cold prostaglandin to enable estimation of recoveries following extraction procedures. Two standard tracer/cold prostaglandin mixtures were made up, one containing 10 μg cold PGE_2 + 2 μCi ^3H-PGE_2 in 1 ml H_2O, and the other containing 10 μg cold $PGF_{2\alpha}$ + 2 μCi ^3H-$PGF_{2\alpha}$ in 1 ml H_2O. 4 x 10 μl of each mixture was sampled into 10 ml toluene scintillant for monitoring of its radioactivity. Each standard tracer/cold prostaglandin mixture was mixed with Dextran-Tyrode solution as follows:

(100 μl tracer/cold mixture → 10 ml Dextran-Tyrode solution) x 3.

The six solutions were then adjusted to pH 4.5 with 10% acetic acid and extracted as usual with 2 x 2 vols redistilled ethyl acetate. The aqueous phase was discarded whilst the ethyl acetate phases were pooled and washed with 1/10 vol H_2O to remove acetic acid. The ethyl acetate was then evaporated to dryness under reduced pressure and the residues desiccated. Residues were dissolved in 1 ml methanol and 4 x 10 μl of each residue was sampled into scintillant for radioactive counting as before. The recovery

of labelled prostaglandin could be determined by comparing radioactive counts obtained pre- and post-extraction. The mean recovery of PGE_2 (from the three tracer/cold mixtures) was 98.3% and that for $PGF_{2\alpha}$ was 105.4%. It was concluded that Dextran-Tyrode solution did not interfere with the extraction of prostaglandins using ethyl acetate. It was decided, however, to check that the radioactivity measured following extraction did, in fact, represent intact prostaglandin. This involved the absolute analysis of the residues by combined GC/MS. The methanolic extracts were transferred to Eppindorf micro-tubes and evaporated almost to dryness in preparation for derivatisation (see Section 1). A residual amount of methanol was retained to catalyse the methylation of samples with diazomethane. Following methylation of acid groups of both PGE_2 and $PGF_{2\alpha}$ samples, PGE samples were oximated (to protect the 9-keto group) by treatment with about 100 µl of methyl methoxime (in pyridine) and incubation at 60°C for 1.5-2 hours. The pyridine was removed by vacuum desiccation. Methyl esters of $PGF_{2\alpha}$ and methyl methoxime derivatives of PGE_2 were then silinated with 50 µl BSTFA (60°C for 15 min.).

10 µl aliquots were injected on to the GC column and mass spectra were taken at appropriate retention times. Positive identification of PGE_2 and $PGF_{2\alpha}$ was obtained with major peaks occurring at m/e values of 295 (M-(199 + 45)) for PGE_2 and 423 (M-(90 + 71)) for $PGF_{2\alpha}$).

From these results it could be inferred that Dextran-Tyrode did not affect either the stability of prostaglandins

or their chromatographic and mass spectrometric behaviour. The possibility remained that other substances released from the kidney into the perfusate might affect analyses of prostaglandins.

Preparation of biological samples

The levels of prostaglandin (if any) in Dextran-Tyrode perfusate from the isolated kidney preparation had not previously been measured. It is desirable to measure peak ratios in triplicate during MID analysis. It was suspected, however, that the prostaglandin contained in a one minute perfusate fraction (10 ml), particularly under resting conditions, might not contain sufficient prostaglandin for multiple injection. Samples were therefore grouped into types according to the timing of their collection relative to RNS:

Pre-stimulation or resting sample	- Minute 1	- Sample R
Stimulation sample	- Minute 2	- Sample S
First post-stimulation sample	- Minute 3	- Sample PS
Second " " "	- Minute 4	- Sample P_2S
Third " " "	- Minute 5	- Sample P_3S

Four resting samples obtained from one experiment and correspondingly four of each other "type" of sample were pooled for prostaglandin analysis. Each group of samples will henceforth be referred to in the singular. The volume of each sample was measured, always being approximately 40 ml (4 x 10 ml/min). Deuterated (d_4) PGE_2 (1 µg) and $PGF_{2\alpha}$ (1 µg) were added to each sample prior to extraction. These

known amounts of d_4, as in standard calibration samples, acted as internal reference standards.

Extraction and derivatisation of calibration standards and biological samples

The following procedures were carried out on all samples (calibration and biological). Samples were acidified to pH 4.5 with 10% acetic acid and were extracted with redistilled ethyl acetate as described for the "mock" analyses. The residues obtained after evaporation were desiccated and re-dissolved in methanol for transfer into Eppindorf tubes. Ethylation was carried out as previously described for methylation, replacing diazomethane with diazoethane (slightly more active). This was followed by oximation of all samples (to protect the 9-keto group of PGE) by incubation with about 100 µl of pentyloxime (in pyridine), at 60°C for 1.5-2 hours. The pyridine was removed by vacuum desiccation and samples were silanated with BSTFA, 25 µl to each biological sample and 50 µl to each calibration sample. After incubation for 15 min at 60°C, samples were supposed to contain the ethyl/TMS derivatives of $PGF_{2\alpha}$ (Mol. Wt. 598) and d_4 $PGF_{2\alpha}$ (Mol. Wt. 602) and the ethyl pentyloxime/TMS derivatives of PGE_2 (Mol. Wt. 596) and d_4 PGE_2 (Mol. Wt. 600) (see Fig. 1i).

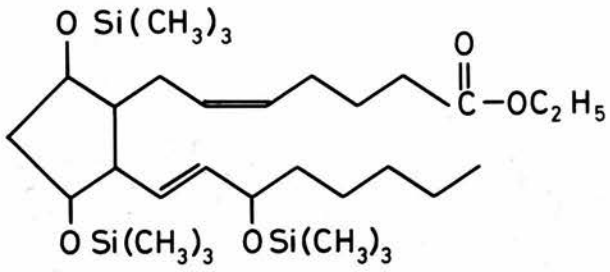
Multiple Ion Detection

The Finnigan 3000D mass spectrometer was capable at the time of analysis of monitoring four ions in parallel.

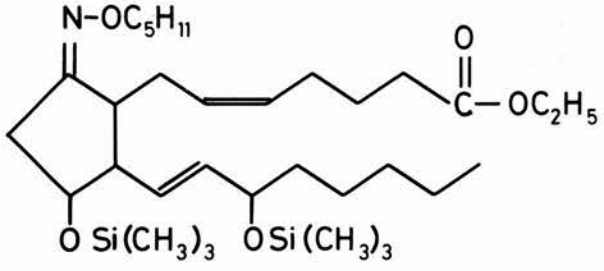
Figure 1i: (1) Ethyl trimethylsilyl $PGF_{2\alpha}$.

(2) Ethyl pentyloxime trimethylsilyl PGE_2.

(1) $PGF_{2\alpha}$ M.Wt. 598



(2) PGE_2 M.Wt. 596



It was necessary, therefore, to tune the machine in such a way that it focused on one peak for derivatives of each of d_4 PGE_2, d_4 $PGF_{2\alpha}$, PGE_2 and $PGF_{2\alpha}$. Major ion peaks are produced at m/e values of 437 and 441 for $PGF_{2\alpha}$ and d_4 $PGF_{2\alpha}$ derivatives respectively, and at m/e values of 309 and 313 for PGE_2 and d_4 PGE_2 respectively. It is obviously preferable to focus on more than one m/e peak for each derivative to ensure absolute identification, but in a parallel estimation such as this it was impossible to do so. The presence of a deuterated standard, however, makes it highly probable that a substance which co-chromatographs with the deuterium and produces a major ion peak four mass units down, is a prostaglandin of the same character.

5 µl aliquots of calibration standards and biological samples in BSTFA were injected on to the column. The column (Temp. 250°C) was packed with 3% OV1 and the pressure of the carrier gas, helium, was 14 psi. Prostaglandin content of biological samples is indicated in Results.

RESULTS

Experiment 1: The effect of clamping the renal artery and vein, as required for renal vein cannulation, on spontaneous output of prostaglandin into renal venous blood.

The purpose of this experiment was to investigate the effect of the clamping procedure necessary for cannulation of the renal vein on the output of prostaglandins from the kidney. Renal ischaemia has been shown to cause release of prostaglandin-like substances in canine renal venous blood (McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee and Ng, 1970). Consequently, it was of considerable importance to determine whether essential dissection procedures could influence spontaneous prostaglandin output and, if so, whether the influence was immediate or delayed. Any such influence would clearly affect the credibility of control sampling shortly after completion of dissection.

Methods

One experimental rabbit (2.3 kg) and two donor rabbits (for blood) were anaesthetised with 25% urethane, 7 ml/kg. A detailed description of the dissection and method of blood collection is given in "General Methods" of this section. Cannulation of the renal vein, which demanded clamping of the renal vein followed by clamping of the renal artery, took 2.25 minutes. This time interval was employed for subsequent clampings throughout the experiment. The first sample was collected 3.5 min after completion of the cannulation procedure and was designated

post-clamp 1. After a 30 min period with no clamping intervention, a "control" sample was collected. Fifteen minutes later, a second clamping procedure was effected and a third sample corresponding to Sample 1, and hence designated post-clamp 2, was collected. Sample 4 was collected 30 min later as another "control" and the final clamping procedure was elicited 15 min later with collection of Sample 5 or post-clamp 3, and a final "control", sample 6.

Prostaglandin content of renal venous blood was analysed by bioassay and combined GLC/MS (see General Methods of this section).

Results

The results are shown in Table 1f. Conclusive identification of PGE_2 and $PGF_{2\alpha}$ in most samples was obtained by combined GLC/MS. Examples of mass spectra are shown in Figs. 1j, 1k and 1ℓ. The amounts of prostaglandins in the first pair of samples (post-clamp 1 and control) though high were closely similar. After the second clamping, renal blood flow was smaller and the output of prostaglandins, especially of PGE_2, was also less. During the collection of sample 3 the systemic arterial pressure fell, possibly due to incompatibility of the donated blood. Blood flow had improved by the time that the control sample (sample No.4) was collected but the prostaglandin levels were now a little lower. The third post-clamping sample (sample No.5) contained similar levels of prostaglandin to the preceding control, but the levels in the final control sample (sample No.6) were less.

Table 1f

EXPERIMENT 1. Amounts of prostaglandin measured in renal venous blood samples collected prior to and following clamping of the renal artery and vein

Sample No.	Time of sampling ⊛ (min)	Arterial B.P. Sys/Dias (mm Hg)	Sample	Volume (ml)	Total Renal Blood Flow (ml/min)	PGE_2	$PGF_{2\alpha}$	PGA_2
						ng/min		
1	5	80/50	Post-clamp 1	45.5	9.1	1541*	512*	< 26
2	35	80/50	Control	45.5	9.1	1545*	575*	< 17
3	55	80/50 to 55/25	Post-clamp 2	21.5	4.3	385*	232*	< 18
4	85	65/40 to 60/40	Control	36.5	7.3	304*	119	< 17
5	105	65/45	Post-clamp 3	26.0	5.2	339*	106*	11
6	135	70/47 to 45/30	Control	25.0	5.0	182	58	11

Prostaglandin levels were estimated by bioassay. Values are corrected for recovery.

⊛ Sampling time was calculated from beginning cannulation of the renal vein. Collection periods were of 5 min duration.

* Prostaglandins were identified by combined GLC/MS.

Figure 1j: Mass spectrometric identification of $PGF_{2\alpha}$ (Me/TMS derivative) in a standard sample (upper spectrum) and in a biological sample (lower spectrum). Peak intensities were measured relative to the major peak, m/e 191, which was taken as 100%.

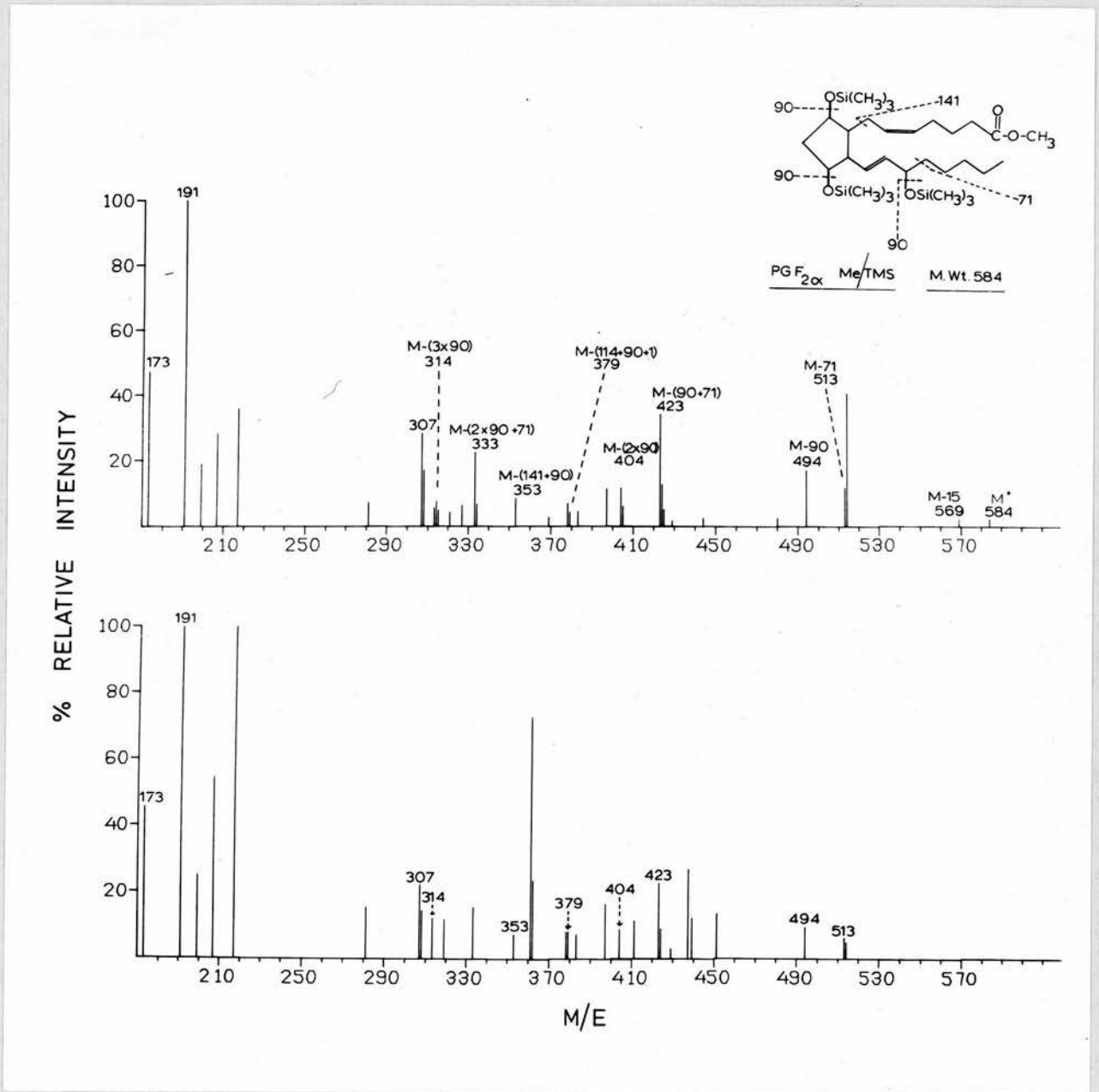


Figure 1k: Mass spectrometric identification of $PGF_{2\alpha}$ (Me/TFA derivative) in a standard sample (upper spectrum) and in a biological sample (lower spectrum). Peak intensities were measured relative to the major peak, m/e 314, which was taken as 100%.

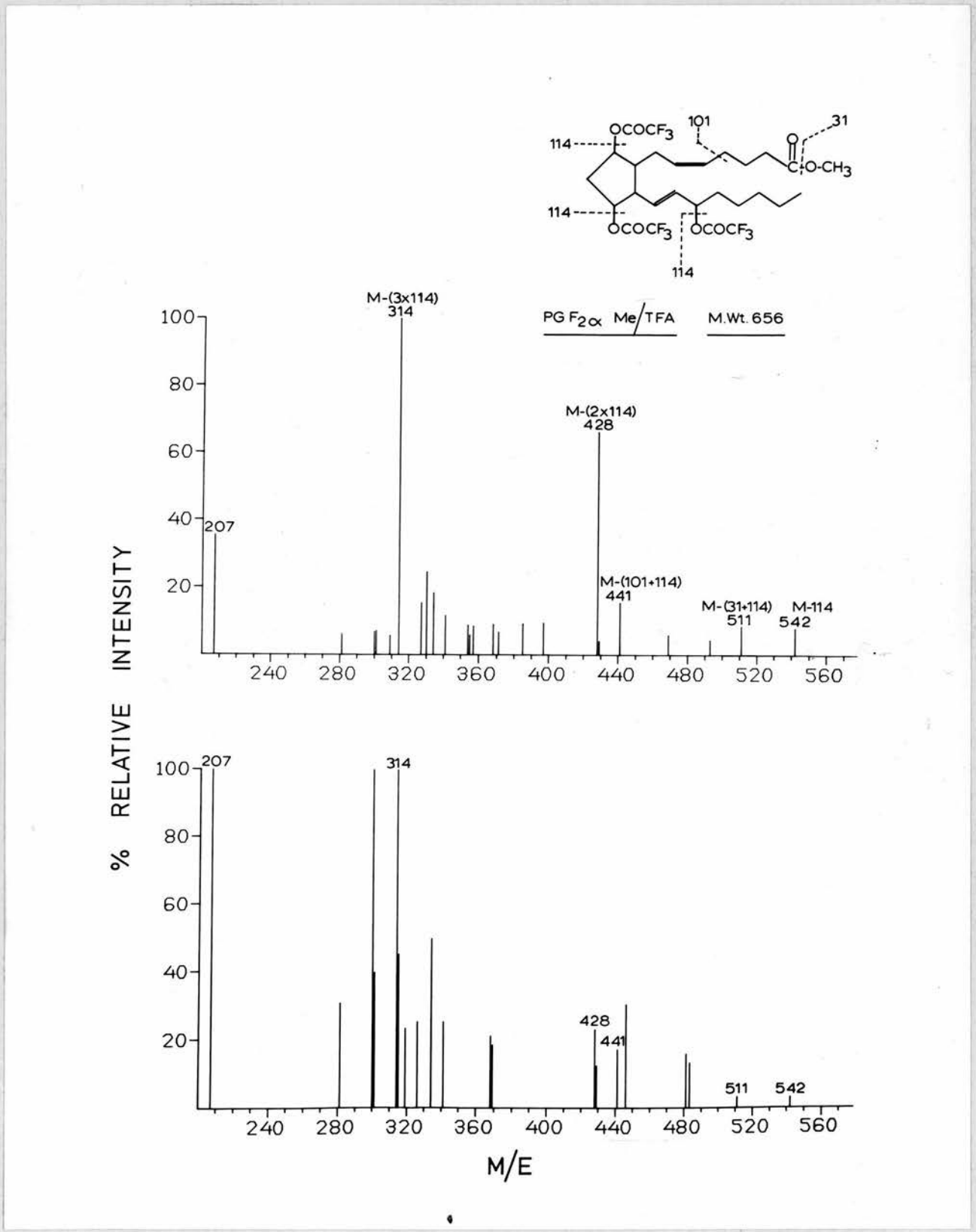
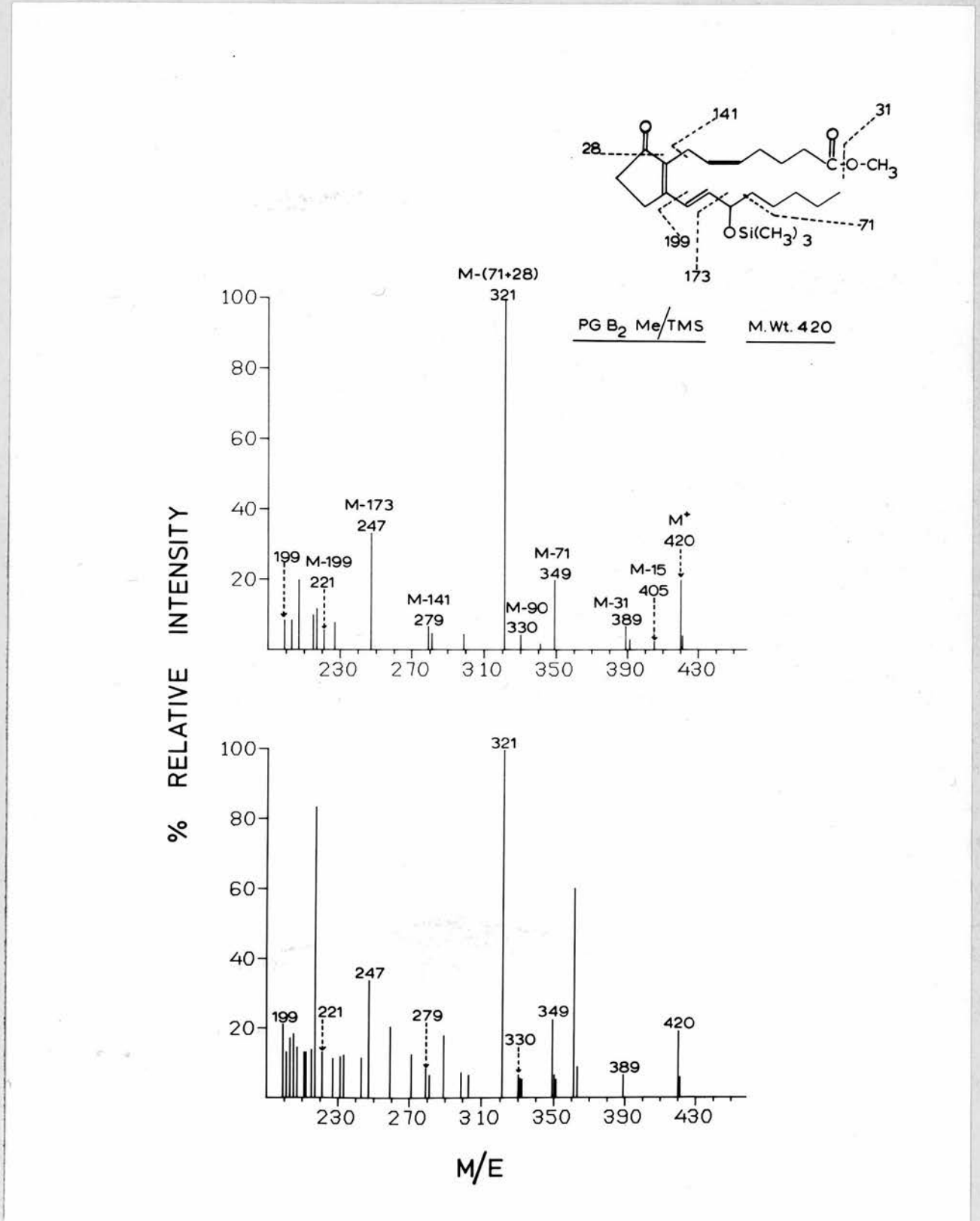


Figure 1ℓ: Mass spectrometric identification of PGB_2 (Me/TMS derivative) in a standard sample (upper spectrum) and in a biological sample (lower spectrum). Peak intensities were measured relative to the major peak, m/e 321, which was taken as 100%.



Conclusion

On the basis of the first pair of samples, it appears that the amount of prostaglandins released from the rabbit kidney is similar whether the collection is preceded by clamping or not. The results of the subsequent clampings are obscured by the decline in prostaglandin output which may reflect a deterioration in the condition of the animal, as suggested by the reduced blood flow and fall in systemic arterial pressure from sample 3 onwards.

In conclusion, rabbit renal venous blood contains high amounts of PGE_2 and $PGF_{2\alpha}$ and a small amount of a PGA, C or B-like prostaglandin, confirming the results of Davis and Horton (1972). It seems probable that the operative procedures in general rather than the clamping procedures caused the large initial output of prostaglandins, since output of prostaglandins fell during the first post-operative hour and then remained relatively constant. The next experiment was designed, therefore, to examine the spontaneous output of prostaglandins over several hours following dissection procedures.

Experiment 2: The spontaneous output of prostaglandins from the rabbit kidney over several hours, keeping body temperature and blood pressure variation to a minimum

Methods

The experimental rabbit was set up as for Experiment 1 and donor blood was collected from two rabbits. Table 1g indicates the timing of sample collection and the systemic arterial pressure recorded during collection. Prostaglandin

content was measured by bioassay and samples were subjected to combined GLC/MS.

Results

Amounts of prostaglandin assayed in renal venous blood are indicated in Table 1g. A gradual increase in prostaglandin output was observed, reaching a maximum in the third and fourth samples. From sample 5 onwards, the output remained relatively stable. The systemic arterial pressure followed an inverse pattern, gradually falling from the time of collection of sample 1 until collection of sample 4. From sample 5 onwards, blood pressure also remained relatively constant. It is interesting to note that maximal output of $PGF_{2\alpha}$ occurred in sample 3, whilst maximal output of PGE_2 was seen in sample 4. This high output of PGE_2 in sample 4 may have resulted from the large fall in arterial blood pressure during collection of this sample, although renal blood flow decreased only slightly.

It seems probable that the increases in prostaglandin output observed in this experiment are explicable in terms of the gradual decrease in systemic blood pressure, leading in turn to a decrease in renal perfusion pressure. The reason for the apparent time displacement of the output curves for PGE_2 and $PGF_{2\alpha}$ is somewhat obscure. It is, nevertheless, an interesting phenomenon which lends support to the idea that the output of each prostaglandin is specifically controlled.

Since the prostaglandin levels in the samples collected were considerably lower in this experiment than in Experiment 1,

Table 1g

EXPERIMENT 2. Amounts of prostaglandin released spontaneously into renal venous blood under control conditions

Sample No.	Time of sampling* (min)	Arterial B.P. Sys/Dias (mm Hg)	Volume (ml)	Total Renal Blood Flow (ml/min)	PGE_2	$PGF_{2\alpha}$	PGA_2
					ng/min		
1	10	90/65	44.0	14.6	26	52	< 25
2	40	80/50	41.5	13.8	84	112	20
3	72	75/45	37.0	12.3	125	256	< 19
4	102	75/45 to 40/20	35.0	11.6	192	170	36
5	132	75/40	34.0	11.3	147	114	23
6	162	75/40	30.0	10.0	167	101	24
7	200	70/45	31.0	10.3	184	105	21
8	230	70/40	32.5	10.8	112	73	21

* Sampling time was calculated from beginning cannulation of the renal vein. Collection periods were of 3 min duration.

Prostaglandin content was estimated by bioassay. Values are corrected for recovery.

mass spectrometric analysis was performed on each PGF fraction (Me/TMS derivatives), on pooled PGE fractions from samples 1-4 and 5-8 (Me/TMS derivatives) and finally on pooled PGA fractions from samples 1-4 and 5-8 (Me/TMS derivatives). There was no confirmed identification of any of the prostaglandins. This was probably attributable to the fact that the amounts of prostaglandin developed were low, often approaching the limit of sensitivity of the mass spectrometer which was 200 ng of each standard prostaglandin. The presence of biological material, even after careful extraction of samples, makes it almost impossible to detect amounts of prostaglandin which are close to the limit of detection of the machine.

Conclusion

The output of prostaglandins from the kidney under the experimental conditions described was variable and difficult to correlate with alterations in either systemic blood pressure or renal blood flow. Prostaglandin levels appeared to stabilise somewhat from sample 5 onwards, however. In order that the effect of drugs (e.g. noradrenaline) on prostaglandin output might be investigated, it would be necessary to begin control blood sampling only after reaching a stable blood pressure. Control and test blood samples should preferably be collected within short time intervals of each other.

Experiments 3 and 4: Effect of intra-aortic noradrenaline infusion on output of prostaglandins from the rabbit kidney in vivo, as measured by bioassay.

When studying the effect of noradrenaline on the output of prostaglandin from the dog kidney, McGiff et al., (1972) infused the drug at concentrations of 50-250 ng/kg/min directly into the renal artery. It was decided, therefore, to utilise a similar dose range in the present experiments, although infusion was in this case intra-aortic rather than renal arterial.

In Experiment 3, doses of 50 and 100 ng/kg/min were tested and in Experiment 4, doses were 200, 500 and 1000 ng/kg/min. Details of collection timing and volumes of blood samples are indicated in Tables 1h and 1i. Plasma levels of prostaglandin were measured by bioassay and some samples from Experiment 4 were submitted to combined GLC/MS. Positive identification of prostaglandin by GLC/MS is indicated in Table 1i.

Results

a) Experiment 3

A summary of results for Experiment 3 is presented in Table 1h. It was desirable to find out whether sub-vasoconstrictor doses of NA would affect prostaglandin release. In Experiment 3, 50 ng/kg/min was sub-vasoconstrictor whilst 100 ng/kg/min produced a slight decrease in renal blood flow. The first infusion of 50 ng/kg/min showed little effect on output of prostaglandins (samples 3 and 4) although a repeat of this dose produced a near doubling of

both PGE_2 and $PGF_{2\alpha}$ output (samples 7 and 8). A final infusion of 100 ng/kg/min, although slightly vasoconstrictor, produced a reduction in prostaglandin output (sample 10). The final post-test sample showed a further reduction in prostaglandin content (sample 11). It is possible, however, that there was an insufficient time interval between the penultimate and final noradrenaline infusion (40 min) which may have led to tachyphylaxis of the renal vasculature to NA. Alternatively, the possibility existed that the higher dose of NA did reduce prostaglandin output.

b) Experiment 4

In an attempt to ascertain whether or not doses from 100 ng/kg/min upwards reduced prostaglandin output from the kidney, three higher doses of NA were infused, as previously described. The results are summarised in Table 1i. Doses of 200 and 500 ng/kg/min caused an increase in the output of PGE_2. During the infusion of 200 ng/kg/min, a maximum output was attained during the first test sample (sample 3). During the subsequent infusion, maximum output occurred in the second test sample (sample 8). An increase in $PGF_{2\alpha}$ output was observed only during the first two NA infusions and in both cases levels were higher in the first test samples, 3 and 7, than in the second test samples, 4 and 8. Infusion of 1000 ng/kg/min NA did not increase the output of either PGE_2 or $PGF_{2\alpha}$ into renal venous blood.

Conclusion

Experiments 3 and 4 indicate that NA is capable of increasing prostaglandin output from the *in vivo* rabbit

Table 1h

EXPERIMENT 3. Amounts of prostaglandin (ng/min) detected in renal venous blood under control conditions and during infusion of noradrenaline

Sample No.	Time of sampling* (min)	Sample	Volume (ml)	Total Renal Blood Flow (ml/min)	PGE_2	$PGF_{2\alpha}$	PGA_2
					ng/min		
1	60	Control	20	8.3	31	96	< 42
2	90	Control	"	7.9	93	101	< 36
3	120	Test 1	"	7.1	90	74	< 32
4	130	Test 1	"	8.8	88	77	< 26
5	145	Post-Test	"	7.0	92	72	< 21
6	175	Post-Test	"	6.9	93	47	< 21
7	185	Test 2	"	6.2	150	79	< 19
8	195	Test 2	"	6.6	70	43	< 20
9	210	Post-Test	"	6.4	94	64	< 19
10	235	Test 3	"	4.4	52	20	< 13
11	255	Post-Test	"	4.6	25	19	< 14

* Sampling time was calculated from beginning cannulation of the renal vein. Samples collected before (Control), during (Test) and following (Post-Test) noradrenaline infusion via the abdominal aorta. In Tests 1 and 2 the rate of infusion was 50 ng/kg/min. In Test 3 the rate of infusion was 100 ng/kg/min. Prostaglandin levels were estimated by bioassay. Values are corrected for recovery.

Table 1i

EXPERIMENT 4. Amounts of prostaglandin (ng/min) detected in renal venous blood under control conditions and during infusion of noradrenaline

Sample No.	Time of sampling® (min)	Sample	Volume (ml)	Total Renal Blood Flow (ml/min)	PGE_2	$PGF_{2\alpha}$	PGA_2
					ng/min		
1	60	Control	20.5	11.7	78	lost	< 15
2	80	Control	"	12.9	127	144	"
3	90	Test 1	"	12.7	625*	431	"
4	98	Test 1	"	8.3	386	274	"
5	113	Post-Test	20.0	9.7	401*	181	"
6	145	Post-Test	"	12.0	336	216	"
7	155	Test 2	"	11.2	423	404	"
8	162	Test 2	"	11.0	441	284	"
9	180	Post-Test	20.5	11.9	297	141	"
10	192	Test 3	19.5	8.1	123	69	"
11	200	Test 3	20.0	9.0	228	56	"
12	215	Post-Test	20.5	9.8	186	76	"
13	230	Post-Test	20.5	8.0	100	44	"

® Sampling time was calculated from beginning cannulation of the renal vein. Samples collected before (Control), during (Test) and following (Post-Test) noradrenaline infusion via the abdominal aorta. Tests 1, 2 and 3 represent infusion rates of 200, 500 and 1000 ng/kg/min respectively. Prostaglandin levels were estimated by bioassay and identified by combined GLC/MS (*). Bioassay values are corrected for recovery.

kidney. It must be pointed out, however, that there was no check that mixing of NA with aortic blood was consistent. Thus the amount of NA entering the renal artery was not necessarily a constant proportion of the intra-aortic dose. The effect of NA on output of prostaglandin-like substances from the dog kidney has also been described as variable, however (McGiff et al., 1972). In their experiments the concentration of NA reaching the kidney was controlled by administering the drug via the renal artery. They found, firstly, that NA produced an increased output of PGE but not of PGF. Furthermore, the increase in concentration of PGE was unrelated to the dose of NA but was inversely related to the plasma renin activity. Plasma renin was considered an index of sodium balance. Thus the release of prostaglandin seems to be influenced by an interplay of several factors. The lack of increase in the output of PGF during NA infusion into the dog kidney may simply be explicable in terms of species variation. Dunham and Zimmerman (1970) showed that the increase in biological activity obtained in renal venous plasma of the dog during RNS was mainly accounted for in terms of PGE. The amount of PGF-like activity was very small. Present results, on the other hand, are consistent with those of Davis and Horton (1972) who found that output of both PGE_2 and $PGF_{2\alpha}$ from the rabbit kidney was increased during RNS.

Experiments 5, 6 and 7: Effect of intra-aortic noradrenaline infusion on output of prostaglandins from the rabbit kidney, in vivo, as measured by radioimmunoassay

These experiments were an extension of Experiments 3 and 4, using radioimmunoassay in place of bioassay. This method of assay permitted frequent collection of small blood samples (3 ml) at regular intervals to monitor more closely the effects of NA on prostaglandin output. The time intervals between sample collections are indicated in Table 1j. A detailed description of radioimmunoassay is presented under General Methods. In Experiment 5, two infusions of NA were made, one at 200 ng/kg/min and the other at 500 ng/kg/min. In both Experiment 6 and Experiment 7, three infusions of NA were made at 200, 500 and 1000 ng/kg/min respectively.

Results

The prostaglandin content of samples from each of the three experiments is summarised in Table 1j. In all three experiments, a clear-cut rise in output of $PGF_{2\alpha}$ during NA infusion was observed. The output of PGE_2 also increased during some infusions of NA but the levels of PGE_2 were considerably lower than those of $PGF_{2\alpha}$, in both control and test samples. It is difficult to understand why the levels of PGE_2 were so low since values from experiments utilising bioassay methods indicated that the efflux of PGE_2 was at least as high and often higher than that of $PGF_{2\alpha}$ at any one time. This anomaly is probably attributable to the fact that chemical conversion of low amounts of PGE_2 to PGB_2

Table 1j Experiments 5, 6 and 7

Amounts of prostaglandin (ng/ml) detected by radioimmunoassay in renal venous blood under control conditions and during infusion of noradrenaline

Time of sampling* (min)	Sample	PGE_2			$PGF_{2\alpha}$			(PGA+PGC+PGB)		
		5	6	7	5	6	7	5	6	7
15	Control	14.2	13.2	3.1	11.9	37.2	16.1	2.5	1.9	-
20	Control	11.8	8.4	5.8	24.9	38.7	17.5	2.8	2.1	2.2
25	Control	8.4	15.2	3.3	13.5	36.7	14.3	2.6	3.4	1.8
28	Test 1	15.8	16.3	3.4	26.7	56.8	17.5	4.4	3.3	4.4
33	Test 1	19.2	12.8	2.3	30.6	43.6	17.3	3.6	2.4	1.5
38	Test 1	21.2	14.4	2.6	25.5	44.0	21.2	3.9	2.7	2.2
43	Post-Test	14.2	8.5	2.7	23.5	41.7	16.5	1.8	2.7	2.0
53	Post-Test	17.2	14.8	3.1	26.8	38.9	23.7	3.8	5.0	1.5
73	Post-Test	18.8	29.4	4.3	24.0	40.9	20.9	3.8	7.3	1.4
78	Test 2	17.4	30.8	6.0	37.5	65.7	35.2	3.7	7.4	1.7
83	Test 2	10.8	18.8	5.2	25.6	42.4	34.1	2.3	8.5	1.9
88	Test 2	9.8	16.4	5.4	18.4	40.0	33.3	1.7	4.4	2.0
93	Post-Test	10.0	13.8	5.1	13.5	26.9	17.9	1.7	5.0	1.5
103	Post-Test		13.9	4.3		24.9	18.8		2.8	1.0
123	Post-Test		11.2	6.5		15.5	16.0		2.2	1.4
128	Test 3		7.3	10.3		21.8	38.2		2.9	2.1
133	Test 3		11.7	8.3		23.0	29.7		2.8	2.4
138	Test 3		11.9	6.9		19.1	28.7		2.0	2.2
143	Post-Test		6.9	5.5		9.9	14.5		1.6	1.7
153	Post-Test		4.5	4.1		7.9	14.0		1.8	2.0
173	Post-Test			4.6			14.2			1.4

Samples collected before (Control), during (Test) and following (Post-Test) noradrenaline infusion via the abdominal aorta. In Test 1 the infusion rate was 200 ng/kg/min, Test 2, 500 ng/kg/min and Test 3, 1000 ng/kg/min.

PGE_2 was assayed as PGB_2. Corrections for conversion of PGE_2 to PGB_2 were made according to conversion of a standard amount of PGE_2 to PGB_2. Values; for $PGF_{2\alpha}$ and (PGA+PGC+PGB) are uncorrected.

* Sampling time was calculated from beginning cannulation of the renal vein. 3 ml blood samples were collected.

is rather unreliable and usually incomplete. Although standard conversions were attempted in parallel with those for biological samples (see General Methods), the background medium used for these standards was arterial rather than venous plasma as it contains little, if any, endogenous prostaglandin. It is possible, therefore, that substances present in the venous plasma interfered with the conversion procedure. The use of tritium-labelled PGE_2 as an internal standard would have provided a suitable check on the extent of conversion to PGB_2 in each sample. However, this procedure would have involved separation of radioactivity by thin-layer chromatography, thus defeating the purpose of the method as a specific and rapid one for dealing with large numbers of samples.

The output of (PGA+PGC+PGB) in each experiment appeared to follow closely that of PGE_2. There is increasing evidence which suggests that these prostaglandins derive solely from PGE_2 during extraction and purification procedures (see General Introduction). The tendency of their release to reflect that of PGE_2 in present experiments supports this view. If not derived from PGE_2, the amounts of (PGA+PGC+PGB) measured were too low to produce any significant effect in themselves.

Conclusion

It is concluded from these three experiments that the output of PGE_2, $PGF_{2\alpha}$ and (PGA+PGC+PGB) can be increased by intra-arterial infusion to the kidney of NA. Radioimmunoassay provides a very satisfactory and sensitive method for

measurement of $PGF_{2\alpha}$, but measurement of PGE_2 by its conversion to PGB_2 would seem to be rather unreliable if exact quantitation is desired. It is proposed that the relative comparison of one sample with another for PGE_2 content is valid but that the actual amounts measured may be inaccurate by a common factor relating to the success of chemical conversion in the presence of a common biological background. It is possible, however, that the low levels of PGE_2 are accurate and that values obtained using bioassay are misleadingly high because of the inherent non-specificity of the method.

Experiments 8, 9, 10 and 11: Effect of RNS on output of prostaglandins in Dextran-Tyrode perfusate obtained from the isolated kidney

In these experiments, perfusate prostaglandin content was measured by MID mass spectrometry. Perfusate was collected in 1 min fractions (10 ml) prior to, during and following RNS (5 Hz, 30 s, 10-15 V). Four samples obtained at the same stage in the stimulation cycle were pooled for each estimate (see"General Methods, MID mass spectrometry: Preparation of biological samples" for full details of procedure).

Results

The results are summarised in Table 1k. It may be observed that PGE_2 and $PGF_{2\alpha}$ output (ng/ml) increased either during or immediately following RNS, in every experiment. In Experiments 8 and 9 (samples obtained from the same kidney),

Table 1k Experiments 8, 9, 10 and 11

Amounts of PGE_2 and $PGF_{2\alpha}$ (ng/ml) measured in Dextran-Tyrode perfusate from the isolated kidney, prior to, during and following RNS

Experiment	Sample	PGE_2	$PGF_{2\alpha}$
8	R	8.2	3.0
	S	24.7	14.0
	PS	10.0	3.2
	P_2S	11.5	1.7
9	R	3.7	lost
	S	8.6	1.8
	PS	9.7	4.4
	P_2S	8.0	2.1
10	R	2.8	4.8
	S	3.8	10.1
	PS	4.2	11.5
	P_2S	4.4	4.6
	P_3S	4.8	6.1
11	R	5.0	11.4
	S	did not derivatise	34.2
	PS	7.5	23.1
	P_2S	6.7	11.9
	P_3S	5.5	7.4

R = 1 min prior to stimulation
S = 1 min during stimulation
PS = 1 min following stimulation
P_2S = 2 min following stimulation
P_3S = 3 min following stimulation

the levels of PGE_2 were approximately twice those of $PGF_{2\alpha}$, whilst in Experiments 10 and 11 (samples obtained from the same kidney but not that used for Experiments 8 and 9), the opposite was observed. This discrepancy between the two kidneys in their ability to release each prostaglandin is difficult to explain since experimental conditions were apparently comparable. Unfortunately time did not permit extension of the investigation to include a larger number of kidneys.

Conclusion

It is concluded that RNS is capable of increasing the output of prostaglandins from the isolated kidney. The absolute levels and the relative increases are comparable to those observed *in vivo* by Davis and Horton (1972). The results provide positive evidence that the isolated kidney is a viable preparation for the further investigation of prostaglandin/adrenergic interactions.

Discussion

Ischaemia and manipulation of organs are known to stimulate prostaglandin release (see General Introduction). The high levels of prostaglandin measured in renal venous blood within an hour or so of completing dissection procedures in Experiment 1 may, therefore, have resulted from the manipulative procedures required for cannulation of the renal vein. The temporary ischaemia caused by clamping the renal artery did not appear to be a contributory factor, as clamping interventions later in the experiment failed to induce a rise in prostaglandin output. Haemorrhagic shock was unlikely to stimulate prostaglandin release since blood withdrawn for analysis was simultaneously replaced by donor blood. In Experiment 2, particular care was taken to minimise manipulation of the kidney and its blood vessels during the cannulation procedure. Samples collected shortly after completion of the procedure contained low amounts of prostaglandin. It was concluded from these two experiments that the experimental animal should be left for at least one hour post-operatively to stabilise prior to collection of control blood samples.

Experiments 3 to 7 have provided evidence that intra-arterial infusion of NA to the kidney increases the output of prostaglandin into renal venous blood. An increased output was not observed during every infusion, however. In some cases this may have been attributable to tachyphylaxis to infused NA. The apparent variations in responsiveness to any one dose of NA may have been due to the variable mixing

of NA with aortic blood before passing into the renal artery. Renal arterial concentrations of NA in these experiments could not, unfortunately, be monitored. Renal perfusion pressure could be gauged by observation of systemic arterial blood pressure. Alterations of renal blood flow could also be detected during collection of blood samples in Experiments 3 and 4. It was impossible to detect such changes in Experiments 5, 6 and 7, however, where blood samples were collected in 3 ml amounts for radioimmunoassay. Increased release of prostaglandin from the kidney did not seem to be directly correlated with reductions in renal blood flow, unlike results obtained in the dog by Herbaczynska-Cedro and Vane (1973). These authors also demonstrated that a reduction in renal perfusion pressure caused release of prostaglandins from the dog kidney. The reductions in perfusion pressure in their experiments were as large as 50-100 mm Hg. In general, the reductions in systemic blood pressure, if any, observed during present experiments were very small and probably did not affect prostaglandin output from the kidney. In one instance, however, there was a fall of about 35 mm Hg during collection of a sample in Experiment 2, which may well explain the increased levels of prostaglandin present in that sample.

Mechanism of prostaglandin release

The present experiments indicate that prostaglandins may be released from the kidney by NA in the absence of any vasoconstriction. Similar results have been obtained in the rabbit heart where the vasoconstrictor effect of NA

was abolished by the α-blocker, phenoxybenzamine (Junstad and Wennmalm, 1973a). In these experiments, NA continued to release prostaglandins. Terashima et al. (1974) have also demonstrated the release of prostaglandins from the dog kidney by NA in the presence of α-blockade by phenoxybenzamine. Other authors, however, have shown that phenoxybenzamine does inhibit NA-induced prostaglandin release from the dog spleen (Davies, Horton and Withrington, 1968; Ferreira and Vane, 1967). The apparent requirement of vasoconstriction for the release of prostaglandins from canine organs is further documented by experiments demonstrating that angiotensin II releases prostaglandin-like substances from the dog kidney (McGiff, Crowshaw, Terragno and Lonigro, 1970b; Aiken and Vane, 1971, 1973) and that both angiotensin II and histamine cause release of prostaglandin-like material from the dog spleen (Ferreira and Vane, 1967). Thus vasoconstriction rather than specific α-receptor activation seems to be the stimulus for prostaglandin release in the dog. It is probable that the various substances which provoke release of prostaglandin, do so by acting on a common biochemical pathway.

In agreement with present results in the isolated kidney (Experiments 8 to 11), Needleman, Douglas, Jakschik, Stoecklein and Johnson (1974a) have demonstrated the ability of RNS to release a prostaglandin-like substance from the isolated Krebs-Henseleit-perfused rabbit kidney. Noradrenaline and adrenaline also stimulated prostaglandin release and the effects of all three adrenergic stimuli were blocked by the

α-receptor antagonist, phenoxybenzamine, indicating that the effects were α-receptor mediated. Angiotensin II infusion and renal ischaemia (for a 4 min period) caused a release of prostaglandin which was unaffected by phenoxybenzamine. The effects of both stimuli were blocked by the prostaglandin synthesis inhibitor, indomethacin, and that of angiotensin II could also be blocked by the specific angiotensin antagonist, cysteine-8-angiotensin II (Needleman, Kauffman, Douglas, Johnson and Marshall, 1973). These results extend the evidence previously discussed that prostaglandin release seems to be multireceptor activated. A common pathway of action via adenyl cyclase/cyclic AMP seems unlikely, however, in view of the findings that cyclic-AMP, dibutyryl cyclic AMP and theophylline were inactive as inducers of prostaglandin release (Needleman et al., 1974a). It should be emphasised that in the foregoing experiments, evidence of prostaglandin release relied solely upon superfusion bioassay. No chromatographic purification or chemical identification of prostaglandins was performed.

The nature of the prostaglandin-release mechanism is evidently complex and the common stimulus, if any, remains to be elucidated.

Site of prostaglandin release

As described in the introduction to this section, the site from which prostaglandins are released into renal venous blood in response to adrenergic stimuli may be either prejunctionally or postjunctionally located, i.e. at

the adrenergic nerve ending or at the effector cell. The experiments from this section point to a probable postjunctional site of release, as both RNS and NA are capable of stimulating the release of prostaglandin. The existence of α-adrenoceptors on the adrenergic nerve ending itself does confuse the argument, however. For this reason, α-blocking drugs were not used in the present experiments as it was thought that their ability to block both pre- and postjunctional adrenoceptors would not clarify the situation. However, more recent evidence indicates that the α-blocking drug phenoxybenzamine can selectively block postjunctional receptors at lower concentrations, up to 2.9×10^{-9}M. A tenfold increase in dose seems to act both pre- and postjunctionally (Dubocovich and Langer, 1974). The possibility still exists, therefore, that NA acts on both pre- and postjunctional receptors and may release prostaglandins from either or both sites. There is one piece of evidence which suggests a prejunctional release of prostaglandin. Stjärne (1972) has demonstrated that when the contractile response of the isolated guinea pig vas deferens is blocked by reducing the external calcium concentration of the bathing medium to a degree which still permits some adrenergic transmitter release in response to field stimulation, the overflow of transmitter is elevated by the addition of a prostaglandin synthesis inhibitor (eicosatetraynoic acid) to the medium. Prostaglandin may, in this case, be controlling transmitter release from a prejunctional site. It is conceivable, however, in the light of the evidence

discussed under "Mechanism of prostaglandin release", that prostaglandin may continue to be releasable from a postjunctional site in the absence of contraction. The evidence against a prejunctional site of release of prostaglandin is quite considerable. Chemical denervation of the kidney to prevent reflexogenic sympathetic stimulation did not modify the increase in output of prostaglandin during reduction of renal blood flow (Herman and Vane, 1974). Similarly, denervation of the rabbit heart did not significantly alter the outflow of PGE_2 in response to NA infusion (Junstad and Wennmalm, 1973a). Further evidence against a prejunctional site of release is the knowledge that prostaglandin release is not dependent upon the activation of adrenergic receptors (by either nerve stimulation or NA). Angiotensin and histamine also stimulate the release of prostaglandins (Aiken and Vane, 1971, 1973; Ferreira and Vane, 1967).

It is concluded that prostaglandins are released in response to various substances which are known to act on different receptors. This would suggest that their release is triggered by an action on some common biochemical pathway at the level of the effector cell. The possibility that some prostaglandin is released from adrenergic nerves during stimulation has not yet been eliminated.

SECTION 2

A comparison of the influence of prostaglandins A_2, E_2 and $F_{2\alpha}$ on vascular responsiveness to noradrenaline and renal nerve stimulation in the rabbit kidney

The ability of noradrenaline to increase prostaglandin output in renal venous blood described in Section 1 indicates that there is some interaction between prostaglandins and the adrenergic system in the rabbit kidney. The present experiments were designed to investigate this interaction from another angle, namely the effect of prostaglandins on responses to adrenergic stimuli. Such interactions have been widely studied in various systems (see Horton, 1969, 1972, 1973; Brody and Kadowitz, 1974; Vane and McGiff, 1975 for references), and are thought to involve two components (i) an action of prostaglandin on the adrenergic transmitter release mechanism, henceforth designated a 'prejunctional' action, and (ii) an action of prostaglandin on the post-synaptic effector cell, or 'postjunctional' action.

In this section an attempt is made to dissociate the possible prejunctional and postjunctional influence of prostaglandins. This involves a comparison of the action of prostaglandins on vascular responses of the kidney to direct (NA) and indirect (RNS) adrenergic stimuli. Emphasis has been placed upon the study of PGE_2 since it is the most abundant renal prostaglandin (see Daniels, Hinman, Leach and Muirhead, 1967; Larsson and Änggård,

1973a, for references) and has been proposed as a regulator of adrenergic neurotransmission in several other tissues and organs.

The ubiquity of $PGF_{2\alpha}$ in many tissues and its ability to be released from them during sympathetic nerve stimulation has led various workers to investigate its interaction with the adrenergic nervous system (Kadowitz, Sweet and Brody, 1971a, 1971b, 1972; Hedqvist and Wennmalm, 1971; Davies and Withrington, 1971; Hedqvist and von Euler, 1972). Since $PGF_{2\alpha}$ is also produced in large quantities by the rabbit kidney (Davis and Horton, 1972; Larsson and Änggård, 1973a), its effects on adrenergic neurotransmission are investigated in this section and in Section 3.

The paucity of information on the interactions of A prostaglandins with adrenergic neurotransmission is surprising in view of its well-established vasodilator activity (see Lee, 1973, for references) and ability to withstand passage through the lungs without undergoing degradation (Horton and Jones, 1969; McGiff, Terragno, Strand, Lee, Lonigro and Ng, 1969). On account of these properties, it has been postulated that prostaglandins of the A series may be circulating anti-hypertensive hormones. The possibility of such a function merited an investigation into the interactions of PGA_2 with adrenergic neurotransmission in the kidney.

The isolated perfused rabbit kidney was used in most experiments, being chosen with a view to its future adaptability for transmitter studies. Some <u>in situ</u> experi-

ments were also conducted, however, as a check on the validity of the in vitro situation.

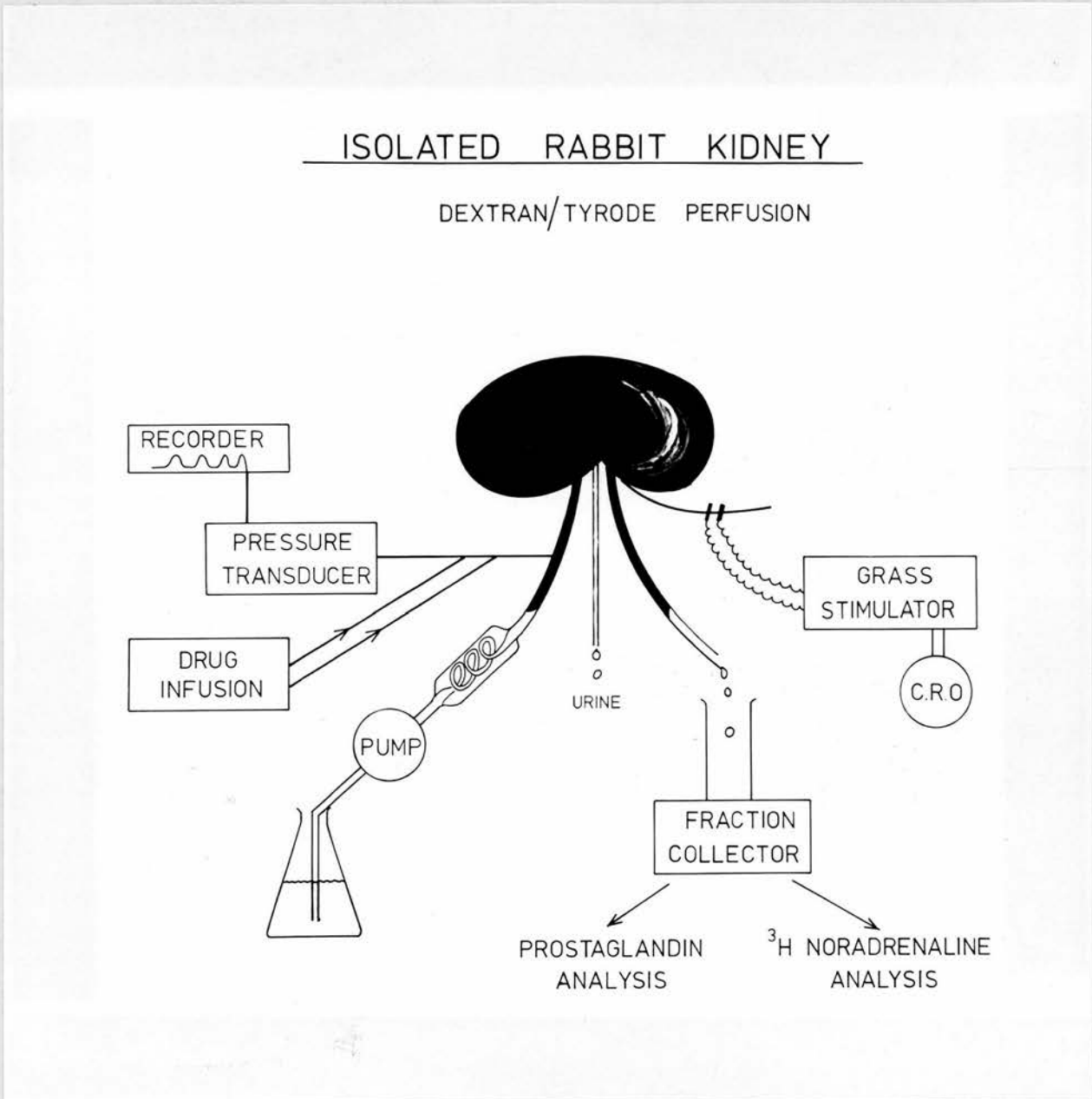
Experiment 1: Effect of PGE_2 on vascular responses of the isolated perfused rabbit kidney to nerve stimulation and noradrenaline infusion

Methods

Twenty rabbits weighing 2.0-2.5 kg were anaesthetised with 25% urethane, 7 ml/kg. The abdomen was opened along its midline and the left kidney exposed. The left kidney was used in preference to the right in all experiments as a result of its anatomical advantages, namely, its longer artery, vein and nerve. The kidney, with its nervous and vascular supply, was dissected free from the surrounding tissue. The nerve was then freed from the artery. After heparinisation of the animal (1 i.u./g body weight) the blood vessels and ureter were cannulated, the nerve was cut and the kidney was flushed with warm 0.9% saline containing 50 i.u. heparin/ml. The preparation was then transferred immediately to a perfusion chamber maintained at 37°C for perfusion with Tyrode solution (mM concentrations: NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$, 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5) also at 37°C, containing 2% dextran (MW 60,000 - 90,000) and gassed with 5% CO_2 in O_2 (see Fig. 2a).

Constant flow perfusion was chosen in preference to the free-flow method to facilitate fractionation of the perfusate for transmitter or prostaglandin analysis. Flow rate was kept at 10 ml/min using a Watson-Marlow pump and

Figure 2a: Diagrammatic representation of the isolated perfused rabbit kidney.



perfusion pressure was recorded on a Grass model 5 polygraph using a Statham (P23AC) pressure transducer.

The nerve was placed on platinum electrodes, protected from drying with Plastibase (Squibb) and stimulated with a Grass S5 stimulator at 10 min intervals.

Changes in perfusion pressure, henceforth called vascular responses, were induced using the following stimulation parameters:

Impulse duration:	2 ms
Stimulation frequency:	8-10 Hz
Voltage:	5-15 V
Period of stimulation:	15-30 s

Vascular responses to NA, comparable to those produced by RNS, were induced by intra-arterial infusion of NA (0.25 - 0.52 µg total) over a 20-30 s interval. PGE_2 (1.5×10^{-8} to 1.5×10^{-6}M) was infused intra-arterially for 2 min prior to and also during RNS or NA administration. Braun-Melsungen pumps were used for all infusions. The effect of PGE_2 on vascular responses to RNS or NA was estimated by calculating the height of a vascular response obtained during PG administration as a percentage of the preceding vascular response. At least two control vascular responses were obtained prior to the administration. If there was a gradual but regular decrease in amplitude of control responses, the vascular response obtained during PG infusion was estimated relative to the predicted control value.

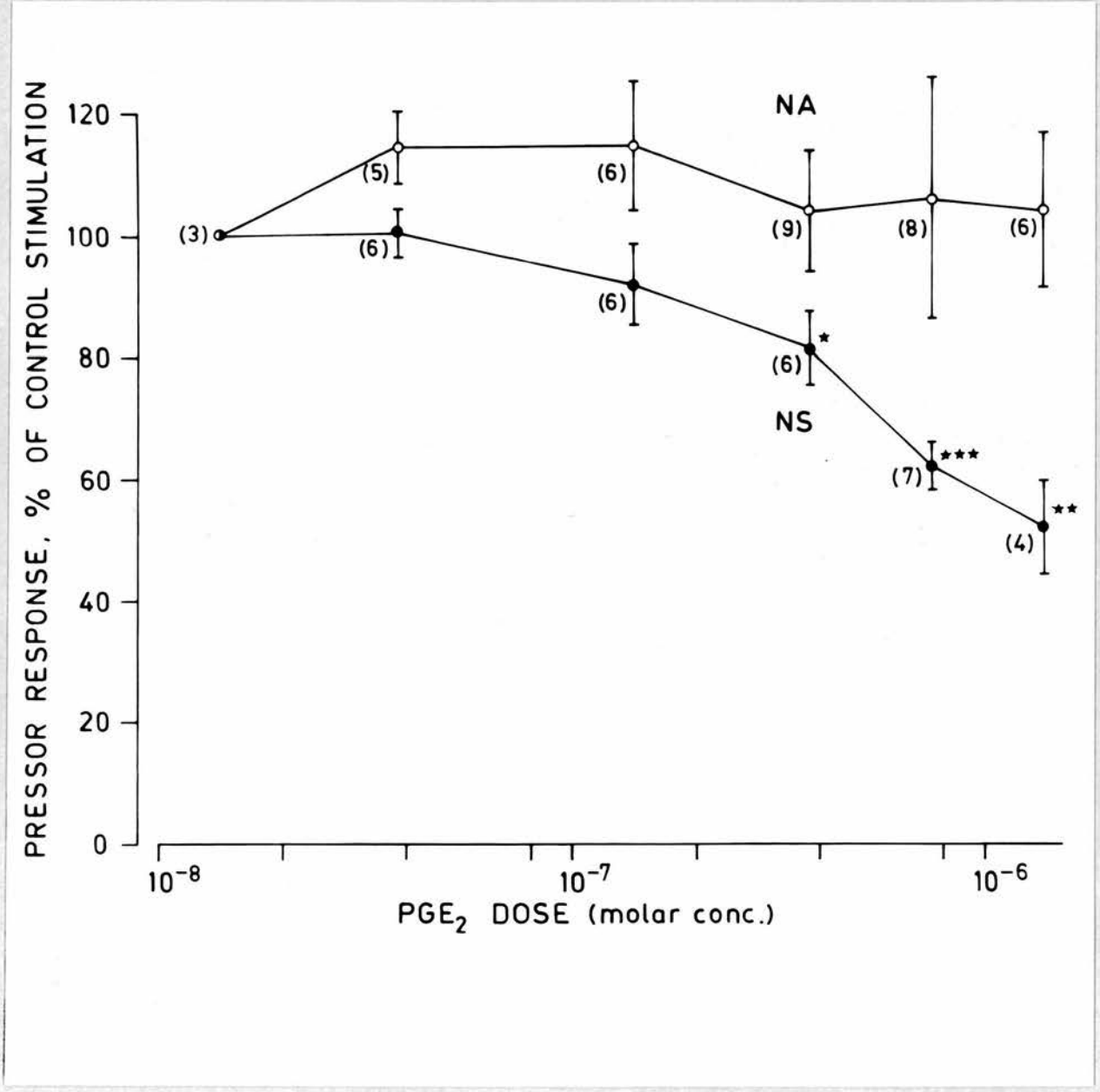
Results

It was consistently observed that concentrations of PGE_2 ranging from 3.8×10^{-7} to 1.5×10^{-6}M, which did not alter basal perfusion pressure on infusion, caused significant inhibition of vascular responses to RNS (NS, Fig. 2d). There was partial or complete recovery from this inhibition 10 to 20 min after ending the infusion of PGE_2. At dose-levels of 1.5×10^{-8} to 3.8×10^{-8}M, PGE_2 showed no significant effect on responses to RNS in most cases, although weak inhibition or even slight enhancement of responses was occasionally observed.

At higher dose-levels, 3.8×10^{-7}M, the inhibitory effect of PGE_2 was found to be statistically significant ($p < 0.05$ to $p < 0.001$), according to Student's t-test for paired variates (see Fig. 2b).

In contrast to its effect on responses to RNS, PGE_2 in the same concentrations did not produce well-defined, dose-dependent effects on vascular responses to NA. The most commonly observed effect was that of enhancement of responses, although in some experiments either no effect or moderate inhibition was observed. The mean values of responses obtained during infusion of different dose-levels of PGE_2 (1.5×10^{-8} - 1.5×10^{-6}M) were all the same as, or slightly greater than, mean control values and a statistical analysis showed that they were not significantly altered from control level by PGE_2 (NA, Figs.2b and 2d).

Figure 2b: Isolated, perfused rabbit kidneys. Effect of different doses of PGE_2 on pressor responses to renal nerve stimulation, NS and to noradrenaline, NA. All values presented as per cent of the preceding control stimulation. Vertical bars = mean values ± S.E.M. Figures within brackets = no. of expts. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ according to Student's t-test for paired variates.



Conclusion

PGE_2 inhibits responses of the *in vitro* rabbit kidney to RNS but its effects, in similar dose-levels, on responses to NA are less well-defined and are statistically insignificant. These results suggest that PGE_2 is producing its inhibitory effect on vascular responses to RNS mainly at a prejunctional level, since its post-junctional effects on NA responses are apparently negligible.

Experiment 2: Effect of PGE_2 on vascular responses of the *in situ* blood-perfused rabbit kidney to RNS and NA administration

Methods

Dissection procedure:

Four rabbits were anaesthetised as described previously and a tracheal cannula was inserted. The femoral artery was cannulated for measurement of systemic blood pressure. The abdomen was opened along its midline and the left renal nerve was freed from the artery. The nerve was tied into a perspex box-electrode. Heparinisation of the animal was maintained by administration of 1 i.u./g body weight every two hours. The right carotid artery was then cannulated. The carotid cannula was linked through a constant-rate pump to the renal artery. The pump rate was adjusted so that the renal perfusion pressure approximated normal systemic pressure (90-110 mm Hg). The use of constant flow again facilitated the administration of drugs at constant concentration. A side-

arm for perfusion pressure measurement and two drug inlets respectively were positioned between the pump and the kidney. The renal perfusion pressure thus took into account any additional pressure resulting from drug infusion.

Experimental procedure:

Vascular responses to RNS were obtained by stimulating the renal nerve at 5-8 Hz and 2 ms duration for 15 s periods. To enable a direct comparison of effects of PGE_2 on RNS and NA responses, an attempt was made to match responses to NA with those to RNS. This involved infusion of a suitable NA concentration over a 15 s period.

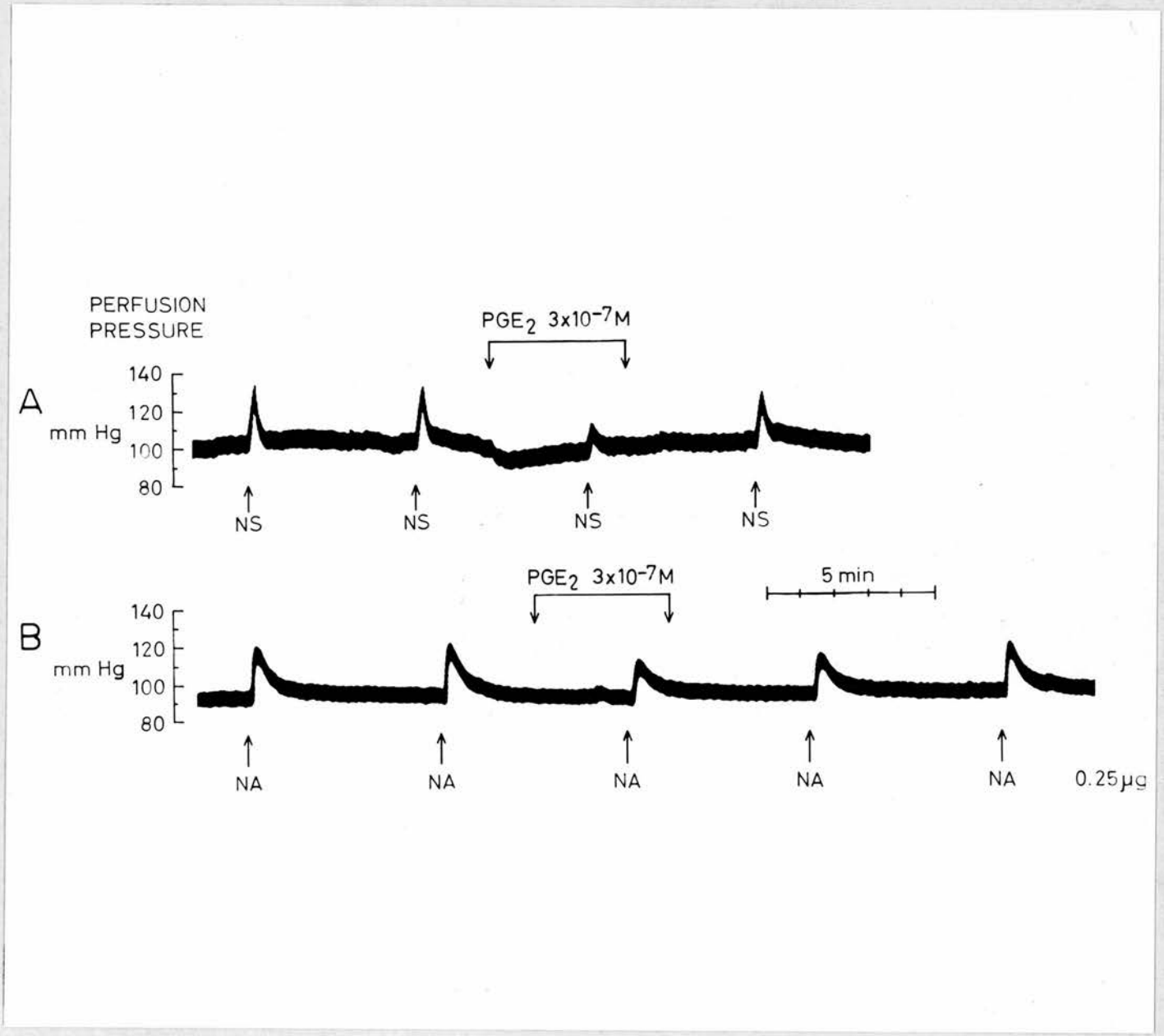
PGE_2 was infused for 3 min prior to RNS or NA infusion and for the duration of the vascular response (about 4 min in total). Both NA and PGE_2 were infused in Tyrode solution at rates not exceeding 0.3 ml/min.

Results

As observed *in vitro*, PGE_2 consistently inhibited vascular responses resulting from RNS, with complete recovery from inhibition occurring about 10 min after ending the PGE_2 infusion. The *in situ* kidney proved more sensitive to the inhibitory effect of PGE_2 than that *in vitro*, marked inhibition of RNS responses being produced even in the lower dose-range, $1.5 \times 10^{-7} - 3 \times 10^{-7}$M (NS, Fig. 2c).

Within the same dose-range, vascular responses to NA were also inhibited by PGE_2, although the inhibition was much less marked than that observed on responses to RNS (NA, Fig. 2c).

Figure 2c: Blood perfused rabbit kidney in situ. Effect of PGE_2 on pressor responses to renal nerve stimulation (A) and to noradrenaline (B) in one and the same experiment.



Conclusion

PGE_2 inhibits vascular responses of the *in situ* rabbit kidney to RNS. There is a quantitative difference in effectiveness of PGE_2 in this preparation when compared with that *in vitro*. The greater sensitivity of the *in situ* preparation may be attributable to the fact that a lower stimulation frequency (5-8 Hz) was required to induce suitable pressor responses than was required *in vitro* (10 Hz). It has been demonstrated that the inhibitory effect on responses to field or nerve stimulation diminishes as the frequency of stimulation is increased (see Section 3).

In contrast to its lack of effect on vascular responses of the *in vitro* kidney to NA, PGE_2 inhibited vascular responses of the *in situ* kidney to NA. The effect was, however, less marked than that produced on responses to RNS. These experiments point to some postjunctional inhibitory action of PGE_2 in the kidney. It is unlikely that such an action can account for the total inhibitory action of PGE_2 on RNS, since the inhibition of NA responses by the same PGE_2 dose-level was approximately half of that observed during RNS.

Experiment 3: Effects of prostaglandins A_2 and $F_{2\alpha}$ on vascular responses of the isolated perfused rabbit kidney to RNS or NA administration

Methods

Twenty-seven rabbit kidneys were set up for isolated perfusion with Dextran-Tyrode solution at 10 ml/min and

perfusion pressure was recorded, as described in Experiment 1.

Vascular responses were induced at 10 min intervals as before by infusing NA over a 30 s period or by stimulating the renal nerve for 30 s (5 Hz, 2 ms duration, 7-15 V).

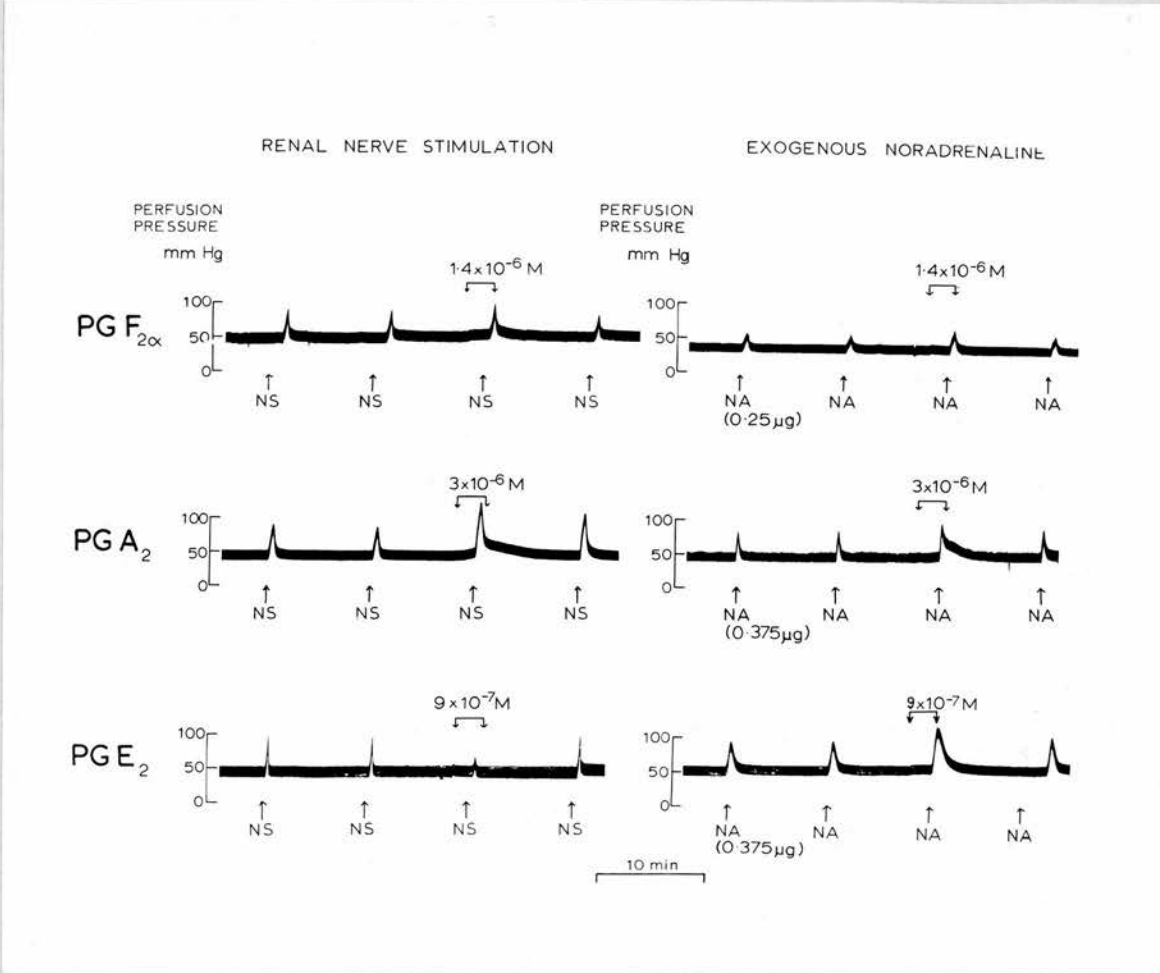
The effect of each prostaglandin on vascular responses to NA and RNS was studied by infusion of the former for 2 min prior to and also during RNS or NA administration (2.5 min infusion). This procedure permitted a direct comparison of results with those obtained in Experiment 1. Drug concentrations were: PGA_2 7.5×10^{-7}M to 3×10^{-6}M; $PGF_{2\alpha}$ 4.2×10^{-7}M to 5.7×10^{-6}M.

Results

The dose-levels of each prostaglandin used in these experiments usually produced a negligible effect on basal perfusion pressure. Occasionally, however, both PGA_2 and $PGF_{2\alpha}$ caused a slight vasoconstriction.

After obtaining a minimum of two control vascular responses to either NA or RNS, prostaglandin was administered as described in Methods. PGA_2 infusion at the above concentrations caused potentiation of vascular responses to both NA and RNS (Fig. 2d). It potentiated vascular responses to NA in four out of five observations at the highest dose-level. In the fifth observation, it had no effect. PGA_2 produced potentiation of vascular responses to RNS in all observations (7) throughout the dose range. $PGF_{2\alpha}$, in the doses indicated, similarly enhanced responses to both NA and RNS (Fig. 2d). Potentiation of four out of five responses to NA and of sixteen out of nineteen responses

Figure 2d: Isolated, perfused rabbit kidneys. Effect of prostaglandins A_2, E_2 and $F_{2\alpha}$ on vascular responses to renal nerve stimulation (NS) and noradrenaline (NA).



to RNS was observed.

An insufficient number of observations was made at each dose-level to allow statistical analysis of the effects of PGA_2 and $PGF_{2\alpha}$.

Conclusion

Since the effect produced by prostaglandins A_2 and $F_{2\alpha}$ was consistent and similar for each type of adrenergic stimulus, it seems probable that these prostaglandins influenced the adrenergic neuro-effector system at a postjunctional level. An investigation into the prejunctional effects of these prostaglandins is required, however, before any final conclusion may be drawn.

Discussion

The results in this section indicate that PGE_2 produces effects on renal vasculature which may be differentiated from those of PGA_2 and $PGF_{2\alpha}$. Furthermore, since the effects of PGE_2 on vascular responses to RNS and NA are quantitatively different, it seems probable that it has two components of action, one prejunctionally and the other postjunctionally located. In contrast, PGA_2 and $PGF_{2\alpha}$ seem to act primarily at a postjunctional level, producing consistent effects on vascular responses to both RNS and NA.

The proposed existence of a prejunctional site of action for PGE_2 in the rabbit kidney is further documented by the work of Malik and McGiff (1975) whose results resemble those found in this study. They found that both

PGE_1 and PGE_2, at infusion concentrations as low as 0.1 ng/ml (sub-vasodilator), inhibited vasoconstrictor responses of the isolated, Tyrode-perfused rabbit kidney to RNS (1-2 Hz) without altering those induced by NA. The extreme sensitivity of the kidney to PGE_2 in these experiments compared with present experiments may be attributable to two factors:

(a) a very low stimulation frequency was employed, at which adrenergic transmission has been shown to be particularly sensitive to PGE (see Section 3); (b) the pressor responses induced were rather small, of the order of 10 mm Hg, such that a 50% reduction in response by PGE_2 would only involve a change of about 5 mm Hg. Pressor responses in the present experiments were considerably larger (20-30 mm Hg). so that changes less than about 2.5 mm Hg were almost certainly overlooked.

In identical experiments carried out on the rat kidney, however, these authors found, curiously, that PGE_1 and PGE_2 potentiated vasoconstrictor responses to RNS (1 Hz) whilst exerting a lesser or no effect on equiconstrictor responses to injected NA. These experiments challenge the extension of the Hedqvist hypothesis to all species, although other results obtained by Needleman, Marshall and Johnson (1974b) using the isolated Krebs-Henseleit-perfused rat kidney indicate general agreement with the hypothesis. Infusion of indomethacin caused potentiation of renal vascular responses to both RNS (3-6 Hz) and NA. In addition, a mixture of PGE_1 and PGE_2, when infused simultaneously with

indomethacin, inhibited vascular responses to RNS. Thus, there is apparent controversy over the intrarenal vascular effects of PGE in the rat kidney. An explanation of the anomaly may be that Needleman et al. used doses of indomethacin which were considerably higher than those employed by Malik and McGiff*: indomethacin 2.5 μg/ml cf. 100 ng/ml respectively; doses of PGE_1 + PGE_2 were lower, however, at 0.5 ng/ml cf. 5 ng/ml. The perfusion media were also different as described, as were perfusion rates (4 ml/min and 6 ml/min respectively) but there is no evidence to suggest that such factors could account for such major differences in the results obtained. Studies of PGE/adrenergic-effector interactions in the kidney have been carried out to a large extent in the dog. Kadowitz et al. (1971c) showed, in agreement with present in situ results, that infusion of PGE_1 (2-4 μg/min) into the in situ, blood-perfused dog kidney reduced vasoconstrictor responses to NA. The effect of PGE_1 on responses to RNS was not examined. McGiff and coworkers have accumulated evidence suggestive of a role for prostaglandins as regulators of the renal actions of pressor hormones in the dog (for earlier references describing release of prostaglandins from the dog kidney and subsequent effects on renal blood flow, see Section 1). They have shown that infusion of PGE_2 into the dog renal artery, in vivo, reversibly inhibits the vasoconstriction/antidiuresis produced by RNS and, to a lesser extent, inhibits that produced by NA. (Lonigro, Terragno, Malik and McGiff, 1973a). These results are compatible with

* See p 153.

results in this section and with the view that PGE-like substances released from the dog kidney on RNS (Dunham and Zimmerman, 1970) or NA administration (McGiff et al., 1972) may serve to attenuate the resultant vasoconstriction, thus allowing eventual resumption of normal renal blood flow. Intra-arterial administration of PGE cannot be equated with its physiological availability, as indicated in Section 4. However, the results discussed do display reasonable parallelism.

Experiments in which $PGF_{2\alpha}$ was administered to the isolated rabbit kidney as in this section, were similarly conducted by Malik and McGiff (1975). They found that $PGF_{2\alpha}$ (5-25 ng/ml) potentiated equi-constrictor responses to RNS (2Hz) and NA, with greater effect on responses to RNS. Higher doses (25-50 ng/ml) also produced a slight direct vasoconstriction on infusion. This is in agreement with the results in this section, although once more there is a large discrepancy in the dose-levels utilised. The possible explanations (a) and (b) advanced to explain discrepancies in PGE concentrations similarly apply here.

In identical experiments on the rat kidney, these authors found that $PGF_{2\alpha}$ did not produce, as one might expect from the results obtained with PGE_2, effects opposite to those in the rabbit kidney, but rather concordant effects.

In a series of experiments in the dog kidney (cf. those done with PGE), $PGF_{2\alpha}$ did not modify vasoconstrictor responses to either RNS (1-6 Hz) or NA. In addition, it did not alter renal blood flow (Lonigro et al., 1973a).

Thus $PGF_{2\alpha}$ shows little potency as a vasoconstrictor/adrenergic modulator in the dog kidney compared with the rat and rabbit.

Contrary to results observed with PGA_2 in this section, Malik and McGiff (1975) obtained results in which PGA_2 produced effects similar to those of PGE_2 in the rabbit kidney; PGA_2 (5-25 ng/ml) reduced vasoconstrictor responses to RNS and to a lesser degree (only at 25 ng/ml) responses to NA. It also caused a fall in basal perfusion-pressure at the high dose. It is difficult to reconcile present results with these and with the accepted vasodilator action of PGA_2. It is conceivable, however, that PGA_2 has a biphasic effect according to dose-level, as has been demonstrated with PGE_2 on pressor responses of the cat spleen, where low doses inhibited and high doses potentiated the responses (Hedqvist, 1970b).

PGA_2 correspondingly mimicked PGE_2 in the rat kidney, producing vasoconstriction and potentiation of responses to RNS and NA. It was again approximately five times less potent than PGE_2 (Malik and McGiff, 1975).

In the dog kidney, PGA_2 also produced effects resembling those of PGE_2 with the equipotent molar ratio, $\frac{PGA_2}{PGE_2}$, of about 5 (Lonigro et al., 1973a).

It is concluded from this section that the discernible selectivity of action of PGE_2 on vascular responses to RNS may assign it a possible role as negative modulator of adrenergic transmitter release in the kidney. The actions of PGA_2 and $PGF_{2\alpha}$ seem to be explicable either in

terms of a direct postjunctional action on the effector cell or in terms of an ability to release adrenergic transmitter. Both mechanisms would account for the direct increase in perfusion pressure and the potentiation effects observed. A knowledge of the prejunctional effects of all three prostaglandins is evidently desirable to clarify present findings. Section 3 comprises such a study.

SECTION 3

A study of the prejunctional effects of prostaglandins A_2, E_2 and $F_{2\alpha}$ on adrenergic neurotransmission in the isolated rabbit kidney

The results in the previous section have provided an indication of the probable site/s of action of each prostaglandin in adrenergic neurotransmission. The inferences, however, were based solely upon observations at the effector cell level. This section attempts to elucidate whether the primary action of each prostaglandin is:

(i) to affect the postjunctional effector cell directly,

(ii) to alter responsiveness of the effector cell to catecholamines (circulating or locally formed),

(iii) to affect prejunctional release of adrenergic transmitter.

This involves an investigation of pre- as well as postjunctional events. PGE_2 has been shown to have a primary prejunctional action on adrenergic transmitter release in various tissues and organs (Hedqvist, 1970a; see also General Introduction for references). The prejunctional actions of prostaglandins A_2 and $F_{2\alpha}$ have not been studied extensively, however.

In this section, the effect of each of the above-mentioned prostaglandins on adrenergic transmitter release in response to RNS is investigated. The overflow of noradrenaline from an adrenergically-innervated organ can be monitored by determining the efflux of tritiated noradrenaline (^{3}H-NA) previously taken up by the nerves

(Hertting and Axelrod, 1961; Rosell, Kopin and Axelrod, 1963; Hedqvist, 1970a; Su and Bevan, 1970; Farnebo and Hamberger, 1971).

General Methods

Labelling Procedure

The isolated rabbit kidney preparation was used in all experiments to facilitate transmitter studies. Kidneys from male or female rabbits (2-3 kg) were set up for perfusion with Dextran-Tyrode solution, as described in Section 2. Radioactive labelling of the noradrenaline stores of each kidney was effected by slow infusion of 25 μCi (1 μCi/min, 7.2 Ci/mmole) of L-[7-^{3}H]-noradrenaline (^{3}H-NA). The ^{3}H-NA was infused in Tyrode solution containing approximately 200 μg/ml ascorbic acid to protect the noradrenaline from oxidation. The kidney was then perfused with isotope-free Tyrode solution for at least 30 min before starting an experiment to remove surplus ^{3}H-NA.

The renal venous cannula was directed into a funnel with the outflow positioned slightly above the kidney to provide a positive venous pressure of about 10-20 mm Hg. The funnel was connected to a 10 ml siphon which emptied into an LKB fraction collector. The rate of perfusion was maintained at 10 ml/min as before, thus providing fractionation into 1 min samples.

The tritium content of samples obtained before, during and after RNS was determined by counting 0.5 ml aliquots in a Packard liquid scintillation spectrometer with quenching monitored by internal standards. Twenty ml of a 3:7 ethanol-

toluene mixture containing 4 g of PPO and 0.1 g of POPOP, per litre of toluene was used as a counting medium for each sample. The efficiency of counting averaged about 45%.

The radioactivity of some perfusate samples was separated into that of intact NA and that of its metabolites by cation exchange column chromatography (Stjärne and Liskajko, 1966; Fredholm and Hedqvist, 1973). In this way it could be decided whether total radioactivity overflowing during RNS was an appropriate indicator of ^{3}H-NA and hence endogenous NA overflow.

Amberlite column chromatography

Cation exchange chromatography of perfusate samples was performed using Amberlite CG-120, Type II (Rohm and Haas, Philadelphia). The Amberlite was placed in a beaker and mixed with water. The water was drained and the Amberlite rinsed in the following cycle: (1) 3 M NaOH containing 1% EDTA (2) H_2O (3) 2 M HCl (4) H_2O (5) 1 M NaAc-buffer, pH 6.0 containing 1% EDTA (6) H_2O. After rinsing, Amberlite was packed to a height of 10 cm in columns of 5 mm internal diameter. The rinsing cycle was then repeated.

After removing 1 ml for radioactive counting, perfusate samples were prepared as follows:

To each sample were added:

10-20 µg ascorbic acid/ml sample

1 µg normetanephrine/ml sample

0.1 - 1% EDTA

1 µg noradrenaline/ml sample

1/10 vol NaAc pH 4.0

The pH of the sample was adjusted to 4.0 with 1 M KOH. The prepared sample was applied to the column and eluted at reduced pressure with the following:

1. 10 ml NaAc pH 6.0 containing 1% EDTA
2. 10 ml H_2O
3. 7 ml 1 M HCl
4. 9 ml 1 M HCl (NA)
5. 1 ml 1 M HCl
6. 1 ml 1 M HCl
7. 10 ml 2 M HCl (Normetanephrine)

The rate of elution was 4 to 5 drops/minute. 1 ml of each effluent fraction (sample effluent + fractions 1-7) was counted in 10 ml Insta-gel (Packard). The values obtained (c.p.m.) were corrected for quenching. The results are shown in Table 3a. The recovery of authentic NA added to the samples and carried through the entire chromatographic procedure was 73.3 $\pm$ 2.3% (mean $\pm$ S.E.M., n = 4).

Since the majority of the radioactivity overflowing in response to RNS ('stimulated') was represented by intact ^{3}H-NA (81.3%), total radioactivity was considered an appropriate indicator of endogenous NA overflow. Conversely, since the majority of the 'background' radioactivity overflowing during resting periods was not intact ^{3}H-NA but rather 'acid metabolites' (74.4%), the amount of ^{3}H-NA overflowing as a result of RNS was calculated by subtracting total background radioactive overflow from total 'stimulated' radioactive overflow.

Table 3a

Cation exchange column chromatography of radioactivity appearing in venous effluent of rabbit kidney previously loaded with ^{3}H-NA

Sample	Number of experiments	'Acid metabolites'	Noradrenaline	Normetanephrine
Stimulated	7	16.8 ± 2.3	81.3 ± 2.2	1.3 ± 0.4
Resting	5	74.4 ± 2.3	14.4 ± 1.1	10.0 ± 1.5

Perfusate samples withdrawn before and during nerve stimulation

Chromatographic values presented as relative distribution (per cent) of recovered 'acid metabolites' (mainly deaminated products), intact noradrenaline and normetanephrine, and given as means ± S.E.M.

Experiment 1: Pattern of adrenergic transmitter overflow during control and stimulation periods

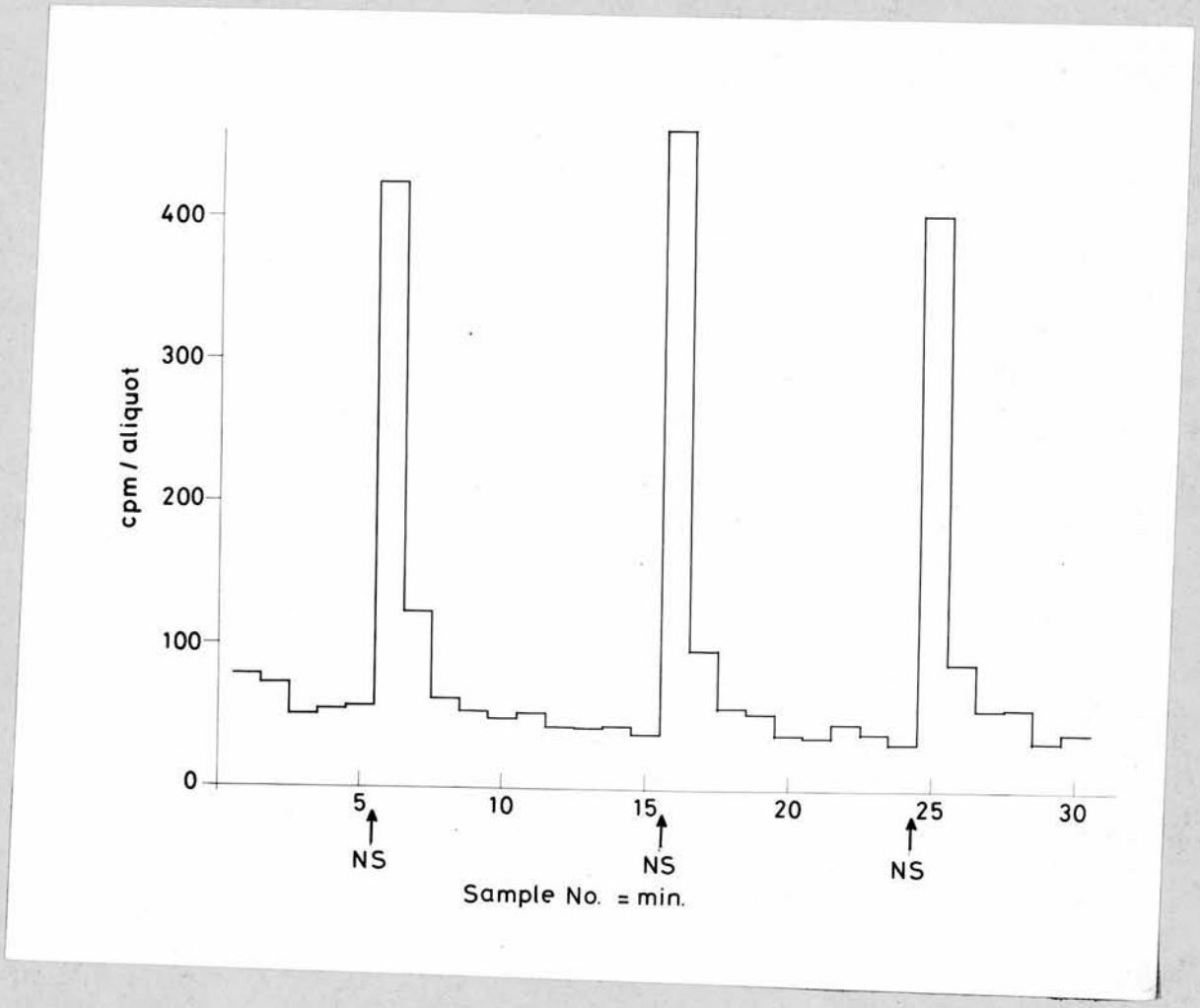
In this experiment the minute to minute overflow of transmitter from the isolated rabbit kidney was monitored, prior to, during and following RNS. This involved sampling every perfusate fraction for radioactive content.

Results

The radioactive overflow during a typical experiment is shown in Fig. 3a. There is a gradual decrease in background radioactive overflow throughout most experiments and a resultant slope in the overflow of radioactivity during RNS. At stimulation frequencies of between 2 and 10 Hz, the ratio $\left(\frac{^3\text{H-overflow during RNS}}{\text{resting } ^3\text{H-overflow}}\right)$ varied between 4 and 20. The total number of pulses was kept constant at 150, regardless of frequency.

At all stimulation frequencies employed, ^{3}H-overflow increased in response to RNS, reaching a maximum within the first or second fraction collected from the onset of stimulation. At the highest frequency of 10 Hz, a maximum was generally reached in the first 'stimulation fraction', i.e. the first fraction collected after the onset of stimulation. At a frequency of 5 Hz, the maximum tended to occur in the second 'stimulation fraction' whilst at the lowest frequency of 2 Hz, the maximum always occurred in the second 'stimulation fraction', the total stimulation time having been extended to 75 seconds. As shown in Fig. 3a, transmitter overflow returns to, or almost to, resting level by the third 'stimulation fraction' (see Fig. 3a, samples 8,

Figure 3a: Isolated, perfused rabbit kidney. Tritium content of perfusate sampled at one minute intervals prior to, during and after renal nerve stimulation (NS).



18, 27 etc.). It was decided, therefore, to calculate the total overflow of tritium resulting from RNS as follows. Radioactive counts from 'stimulation fractions' 1 to 3 were summed and background counts were subtracted:

Total stimulation-induced overflow of tritium

= (stim. fractions 1+2+3) - (3 x pre-stim. background)

This procedure permitted a more accurate assessment of tritium overflow resulting from RNS since it eliminated the effect of a decreasing background overflow.

Conclusion

At stimulation frequencies from 2-10 Hz, the maximum increase in tritium overflow in response to RNS always occurred within the first two 'stimulation fractions'. Furthermore, tritium overflow always returned to background level by the fourth 'stimulation fraction'. It was concluded that an accurate estimate of transmitter overflow per stimulation period could be obtained by sampling two background fractions prior to RNS and three 'stimulation fractions' from the onset of RNS.

In any experiment where a drug was infused for 2 min prior to RNS, its effect on background ^{3}H-overflow was checked by sampling a background fraction prior to drug infusion in addition to the standard sampling described above.

Experiment 2: Effect of exogenous PGE_2 on transmitter overflow in response to RNS

In this experiment, the action of PGE_2, at three dose-levels, on transmitter overflow resulting from RNS was examined.

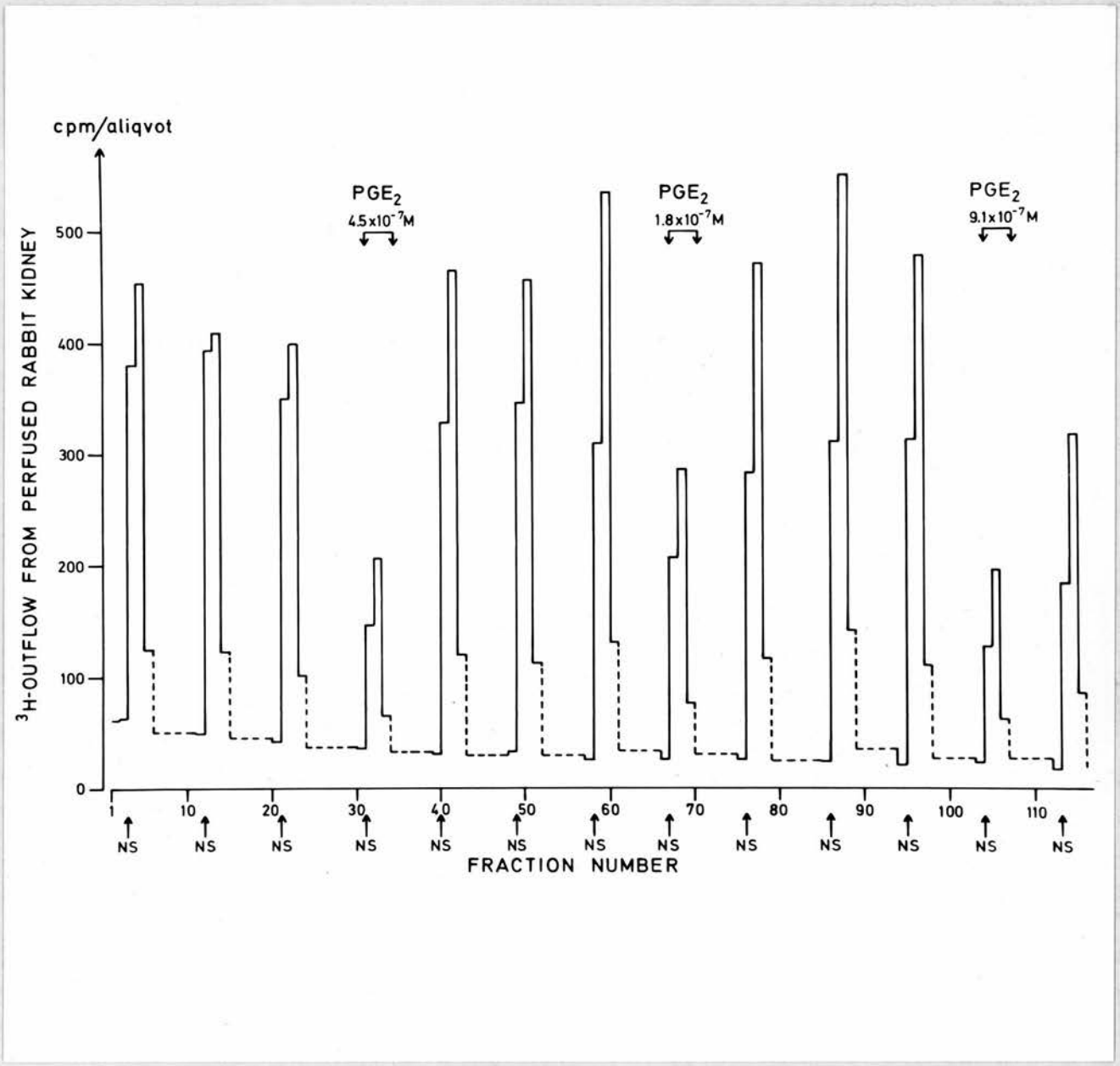
Methods and Results

Thirteen rabbit kidneys were set up for isolated perfusion with Dextran-Tyrode solution at 10 ml/min, as described in Section 2. Each kidney was labelled with 25 μCi ^{3}H-NA (see General Methods) and the renal nerve was stimulated for 15-20 seconds every 10 min at frequencies ranging from 8-10 Hz (2 msec duration, 7-15 V, 150 pulses). PGE_2 was infused intra-arterially to the kidney in Dextran-Tyrode solution at concentrations ranging from 1.8 to 9.1 x 10^{-7}M.

Perfusate was collected for liquid scintillation counting and the total tritium overflow resulting from RNS calculated (see General Methods) for each stimulation period. At least two control stimulation periods were allowed prior to application of PGE_2. The effect of the drug on transmitter overflow in response to RNS was calculated by expressing transmitter overflow during PGE_2 administration as a percentage of preceding control overflow, with the proviso that the two preceding controls had shown regularity in their vascular responses and tritium overflow.

It was consistently found that infusions of PGE_2 (1.8 to 9.1 x 10^{-7}M), for 2 min prior to and also during RNS, decreased the overflow of transmitter in the 'stimulation' perfusate in a dose-dependent manner (Fig. 3b, Table 3b). At these dose levels PGE_2 did not alter basal arterial pressure, nor did it affect 'resting' transmitter overflow. Vascular responses to RNS were similarly inhibited by PGE_2, as previously observed in experiments of Section 2. Partial or complete recovery from both ^{3}H-NA overflow and vascular

Figure 3b: Isolated, perfused rabbit kidney. Effect of PGE_2 on overflow of tracer resulting from renal nerve stimulation (NS). Time in min = fraction numbers. Dotted lines represent uncollected samples.



inhibition occurred about 10 min after ending the infusion of PGE_2.

Table 3b

Percentage inhibition of transmitter overflow to RNS (8-10 Hz, 150 pulses) by PGE_2.

PGE_2 concen.	Percentage Inhibition
1.8×10^{-7}M	18.1 ± 2.3 (n = 9)
4.5×10^{-7}M	29.1 ± 3.3 (n = 9)
9.1×10^{-7}M	30.6 ± 2.9 (n = 8)

Mean values ± S.E.M. figures within parenthesis = number of observations.

Conclusion

PGE_2 inhibited the overflow of transmitter resulting from RNS. Its effect was dose-dependent, although the log dose-inhibition curve obtained was rather flat and appeared to be approaching a maximum inhibition at 30.6%. Since it has been observed in previous studies that the inhibitory effect of PGE_2 is reduced at higher frequencies of sympathetic nerve stimulation (10 Hz) (Baum and Shropshire, 1971; Junstad and Wennmalm, 1973b), the influence of frequency on the PGE_2-log dose/inhibition curve was investigated.

Experiment 3: Influence of stimulation frequency on the inhibitory effect of PGE_2

It has previously been shown in the rabbit heart (Junstad and Wennmalm, 1973b) that the sympathetic transmitter-release mechanism becomes less sensitive to exogenous PGE_2 as the frequency of stimulation is increased from 2 to 10 Hz. In those experiments, however, the number of pulses was not kept constant for the two frequencies but rather the length of the stimulation period, which was 20 seconds. In the present frequency study, it was thought preferable to keep the total number of pulses/stimulation period constant, regardless of frequency. Thus periods of stimulation ranged from 15-75 s. Brown and Gillespie (1957) have shown that noradrenaline release from the cat spleen is almost complete within 20 s of ending sympathetic nerve stimulation (20 s at 10 or 30 Hz). It was assumed, therefore, that measurement of transmitter overflow for 3 min from the beginning of a 15-75 s stimulation period would be adequate for collection of all the transmitter resulting from RNS.

Methods

Twelve rabbit kidneys were set up for isolated perfusion and transmitter collection precisely as in Experiment 2. The renal nerve was stimulated at 2 Hz for 75 s or 5 Hz for 30 s, to enable comparison of the results with those in Expt 2, i.e. the total number of pulses was maintained at 150.

PGE_2 was infused in concentrations equivalent to those in Experiment 2, 1.8 to 9.1×10^{-7}M, for 2 min prior to and

also during RNS. The variation in PG infusion times resulting from corresponding variation in stimulation periods was assumed to be unimportant since the PGE_2 concentration and the rates of infusion were kept constant for all frequencies.

Results

The degree of inhibition of transmitter overflow resulting from RNS (calculated as described in Experiment 1) produced by a given dose of PGE_2 was found to vary inversely with the stimulation frequency employed. The results are shown, together with those from Experiment 2, in Table 3c.

Figure 3c shows a plot of log dose E_2 (abs) versus percentage inhibition of transmitter overflow (ord), at the three stimulation frequencies. The curves for the higher frequencies, 5-10 Hz, appear to flatten off with increasing concentration of PGE_2, suggesting that the inhibition is approaching a maximum. The curve for 2 Hz, although approaching a maximum, does not flatten to the same degree.

Conclusion

These results suggest that an endogenous prostaglandin-mediated braking mechanism would operate more readily at low than at high impulse activity.

Experiment 4: Effects of prostaglandins A_2 and $F_{2\alpha}$ on transmitter overflow resulting from RNS

The previous section demonstrates that prostaglandins A_2 and $F_{2\alpha}$ have a potentiating effect on vascular responses of the isolated kidney to both RNS and NA. Since the degree

Table 3c

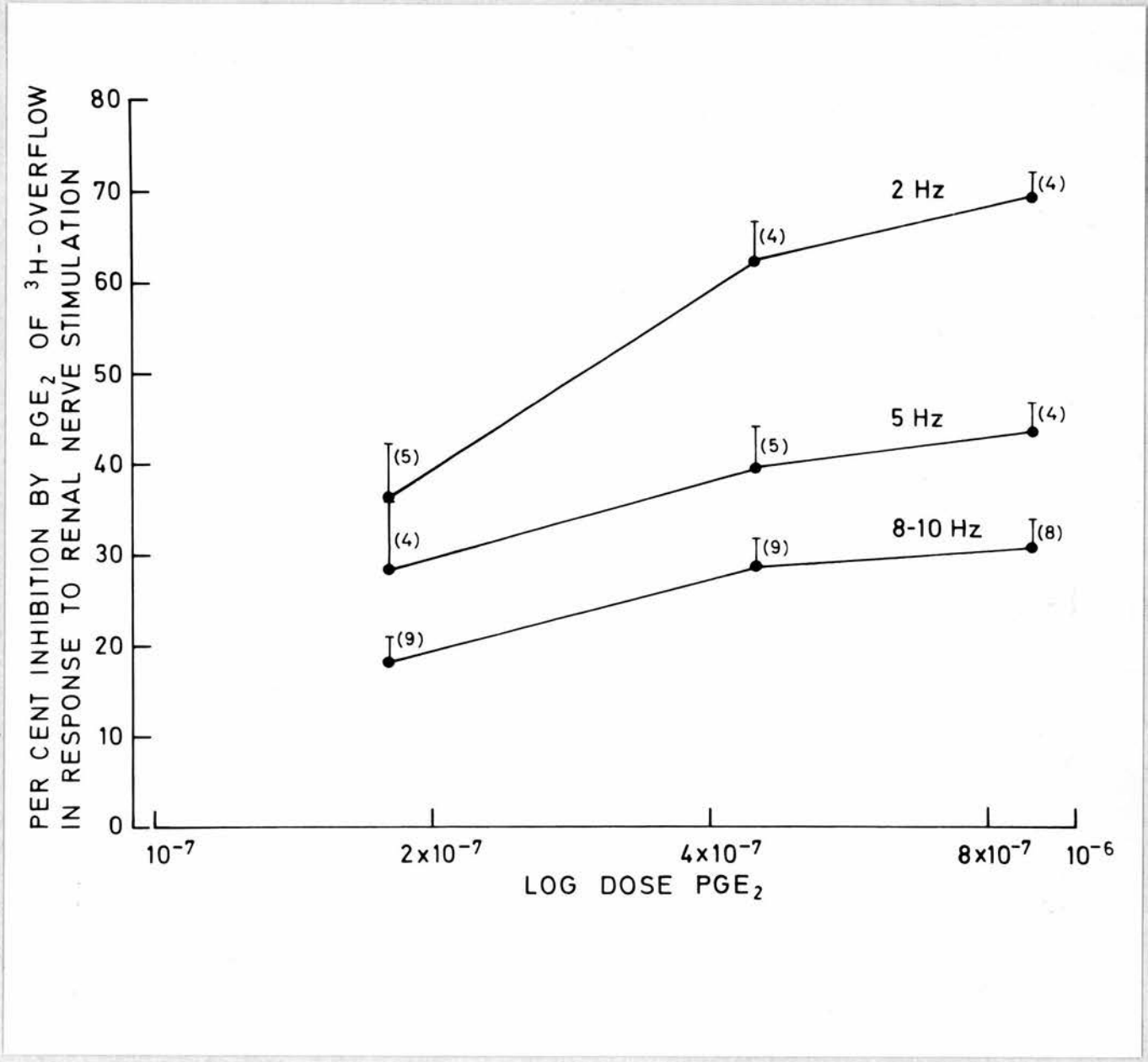
Percentage inhibition by PGE_2 of transmitter overflow resulting from RNS at three different stimulation frequencies

PGE_2 concen. / Stim Frequency	1.8×10^{-7}M	4.5×10^{-7}M	9.1×10^{-7}M
2 Hz (75 s)	36.3 ± 5.7	62.3 ± 4.7	69.2 ± 2.8
	NS	p**	p***
5 Hz (30 s)	28.5 ± 7.7 p**	39.6 ± 4.5 p***	43.5 ± 3.2 p***
	NS	NS	p*
8-10 Hz (15-20 s)	18.1 ± 2.3	29.1 ± 3.3	30.6 ± 2.9

Mean percentage ± S.E.M. * $p < 0.5$ ** $p < 0.01$ *** $p < 0.001$

according to Student's t-test for unpaired variates

Figure 3c: Isolated, perfused rabbit kidney.
Inhibitory effect of PGE_2 on tritium overflow resulting from renal nerve stimulation (RNS), 150 pulses, 2-10 Hz. Vertical bars = mean values ± S.E.M.
Figures at points = number of observations.



of potentiation is similar for each type of adrenergic stimulus, it seems probable that these prostaglandins influence the adrenergic neuro-effector junction at a post-junctional level. A pre-junctional action on the transmitter release mechanism or on the re-uptake mechanism cannot be excluded, however. These possibilities are investigated in the present series of experiments on the isolated rabbit kidney.

Methods

Twenty-five rabbit kidneys were set up for isolated perfusion with Dextran-Tyrode solution as in Section 2. Labelling was carried out as described in this section. In choosing a suitable stimulation frequency with which to activate the renal nerves in these experiments, the criteria were that it should provide reasonable vascular responses and yet be fairly sensitive to the PGE_2-braking effect. A frequency of 5 Hz (2 ms, 7-15 V) fulfilled these requirements, total number of pulses being maintained at 150 as in all previous experiments. Perfusate was monitored for tritium content, as described in Experiment 1 and, after a minimum of two control stimulation periods, prostaglandins were infused as previously, for 2 min prior to and also during RNS. Concentrations employed were: PGA_2, 7.5×10^{-7} to 3×10^{-6}M; $PGF_{2\alpha}$, 10^{-6} to 5.7×10^{-6}M.

Results

PGA_2, infused at concentrations ranging from 7.5×10^{-7}M to 3×10^{-6}M, produced a dose-dependent inhibition of transmitter overflow resulting from RNS (Table 3d). This

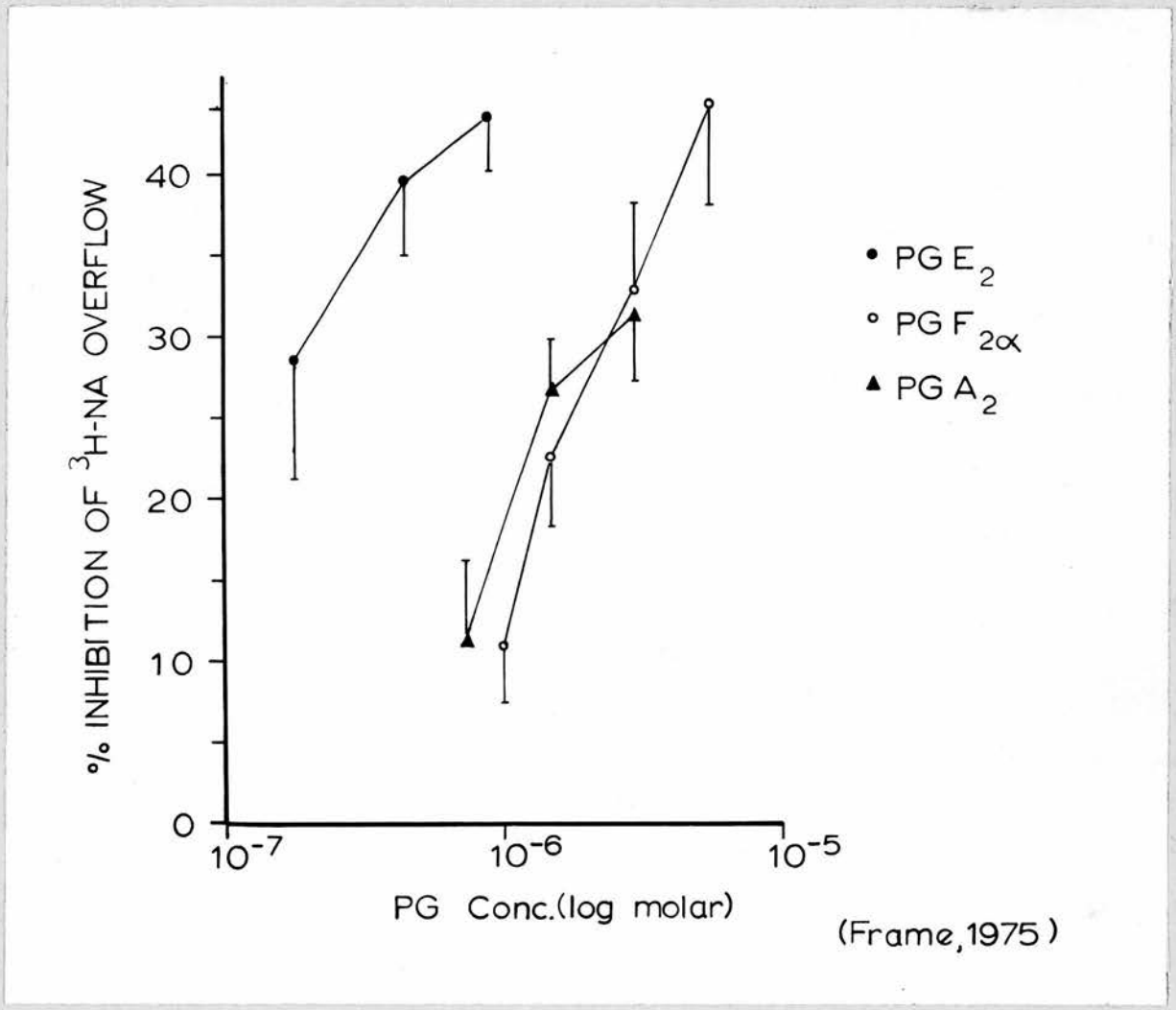
Table 3d

Percentage inhibition of transmitter overflow to RNS (5 Hz, 150 pulses) by PGA_2 and $PGF_{2\alpha}$.

PGA_2 Concentration (M)	% Inhibition	$PGF_{2\alpha}$ Concentration (M)	% Inhibition
7.5×10^{-7}	12.2 ± 5.1 ($p < 0.05$)	10^{-6}	10.9 ± 3.6 ($p < 0.05$)
1.5×10^{-6}	26.6 ± 3.2 ($p < 0.005$)	1.4×10^{-6}	22.5 ± 4.1 ($p < 0.01$)
3×10^{-6}	31.2 ± 4.0 ($p < 0.001$)	2.8×10^{-6}	32.8 ± 5.4 ($p < 0.01$)
		5.7×10^{-6}	44.3 ± 6.3 ($p < 0.001$)

Mean values $\pm$ S.E.M. Significance according to Student's t-test for paired variates.

Figure 3d: Isolated, perfused rabbit kidneys. Effect of prostaglandins A_2, E_2 and $F_{2\alpha}$ on overflow of tritiated noradrenaline (^{3}H-NA) resulting from renal nerve stimulation. Vertical bars = mean values ± S.E.M.



was a surprising observation in view of the simultaneous potentiation of vascular responses (Fig. 2d). The effects of $PGF_{2\alpha}$ on transmitter overflow were comparable to those of PGA_2. There was a dose-dependent inhibition of transmitter overflow (Table 3d), accompanied by a concomitant potentiation of vascular responses to RNS.

Figure 3d shows the log dose/inhibition curves for A_2 and $F_{2\alpha}$ with that of E_2 included for comparison.

Conclusion

It is apparent from these experiments that transmitter overflow resulting from RNS is dose-dependently and reversibly inhibited by both PGA_2 and $PGF_{2\alpha}$. Their potency is, however, only about one-tenth of that of PGE_2, on an equimolar basis (Fig. 3d).

Discussion

(i) Transmitter labelling

It is generally found that tissues and organs in which the concentration of endogenous catecholamines is high are also those which show the highest degree of uptake of tritiated catecholamines. The kidney is capable of binding catecholamines (Axelrod, Weil-Malherbe and Tomchick, 1959; Inouye and Tanaka, 1964) and preferentially noradrenaline (demonstrated with ^{3}H-NA; Whitby, Axelrod and Weil-Malherbe, 1961). This preference for binding of ^{3}H-NA is useful for transmitter studies over long periods of time since the organ is capable of taking up a large amount of ^{3}H-NA. The uptake is selectively into sympathetic nerve

endings since chronic denervation of sympathetically-innervated structures decreases their ability to take up administered ^{3}H-NA (Hertting, Axelrod, Kopin and Whitby, 1961).

The radioactivity emanating from ^{3}H-NA-labelled organs, either spontaneously or as a result of NS, may be separated by cation exchange chromatography into ^{3}H-NA and its metabolites (Stjärne and Lishajko, 1966). The major metabolites are the 3-O-methylated product, normetanephrine and the deaminated or 'acid metabolites', ^{3}H-3-methoxy-4-hydroxymandelic acid (VMA) and ^{3}H-3,4-dihydroxymandelic acid (DHMA).

The results of cation-exchange chromatography of perfusate samples from experiments in this section showed that output of intact ^{3}H-NA increased considerably from 14.4% to 81.3% of the total radioactivity during RNS. In contrast, the acid metabolite content of the perfusate decreased from 74.4% to 16.8% during RNS. Normetanephrine content also decreased from 10.0% to 1.3%. These results are in good agreement with those obtained by Hedqvist, Oliverio and Stjärne (1968) in the dog spleen, where ^{3}H-NA accounted for more than 80% of the radioactivity during NS and less than 10% during resting periods. In other tissues such as adipose tissue (Fredholm and Hedqvist, 1973), skeletal muscle (Rosell et al., 1963), the cat spleen (Hertting and Axelrod, 1961) and the cat nictitating membrane (Langer, 1970), an increase in ^{3}H-NA overflow was observed during nerve or field stimulation, although it made up only 35-50%

of the total radioactivity. In the last two studies there was also an increase in the normetanephrine content of the radioactivity. This indicates that there was high catechol-O-methyl transferase (COMT) activity, degrading the intact ^{3}H-NA. The escape of ^{3}H-NA from renal COMT activity in the present experiments may be explicable by the constant-flow perfusion which would enable an effective washout of any ^{3}H-NA overflowing, hence reducing its availability for degradation.

(ii) Presynaptic effect of PGE

The present experiments clearly demonstrate that PGE_2 inhibits adrenergic transmitter release from renal nerve endings. Prior to this study, the presynaptic actions of PGE's in adrenergic transmission have been investigated largely outwith the kidney, in the cat spleen, guinea-pig vas deferens, rabbit heart and in canine subcutaneous adipose tissue.

In early experiments on the isolated cat spleen (Hedqvist, 1970b), the technique of transmitter labelling was employed, allowing measurement of perfusate radioactivity prior to and during splenic nerve stimulation. The radioactivity was not split into intact ^{3}H-NA and its metabolites but instead a parallel fluorimetric determination of perfusate NA content was carried out, as described by von Euler

and Lishajko, 1961. PGE_2 (6×10^{-8} to 6×10^{-6}M) caused a closely parallel reduction in the overflow of radioactivity and fluorimetrically-determined NA resulting from splenic nerve stimulation, indicating that the metabolic degradation of NA was not apparently altered. This is in agreement with present results in the kidney where, although the ^{3}H-NA overflow in the perfusate decreased during PGE_2 administration, so did the overflow of metabolites, such that the distribution of ^{3}H-NA and its metabolites on a percentage basis was not altered. The uptake of exogenous NA by the spleen and thus probably also re-uptake of endogenously released NA, was not affected by PGE_2, which suggested that its inhibitory effect was not the result of facilitated re-uptake of released NA. Hedqvist concluded that PGE_2 actually depressed the release of NA from the nerve terminals. The highest concentration produced as much as 80% inhibition of NA overflow resulting from NS and this effect was antagonised by increasing the calcium concentration of the perfusate. Thus it was postulated that PGE_2 might act by immobilising the calcium which is required for adrenergic transmitter release (Hedqvist, 1970c).

The effect of PGE_1 on adrenergic transmitter overflow from the isolated cat spleen during NS was less well-defined. Concentrations of 3.8×10^{-8} to 3.8×10^{-6}M were found to inhibit overflow in 4 out of 10 experiments, whilst having no effect in the other 6 experiments (Hedqvist and Brundin, 1969). PGE_1 was not utilised in the present experiments since only the 2-series of prostaglandin types have been conclusively identified in renal venous effluent or in renal

homogenates.

When PGE_2 was added to the superfusion fluid of the isolated guinea-pig vas deferens in concentrations as low as 3×10^{-9} to 3×10^{-8}M, overflow of ^{3}H-NA in response to field stimulation was reversibly and dose-dependently inhibited (Hedqvist, 1974b). This inhibitory effect of PGE_2 was similarly observed in present kidney experiments although at considerably higher concentrations (10-100 fold). The requirement of such high doses is probably explained by the high capacity of the renal cortex for enzymatic degradation of prostaglandins by the enzyme 15-hydroxy prostaglandin dehydrogenase. Intra-arterial administration of prostaglandin to the kidney does not mimic the <u>in vivo</u> situation with the result that penetration to the main site of action may prove difficult. In the vas deferens, on the other hand, penetration difficulties are minimal. PGE_1 in similar concentrations produced closely similar effects on the vas deferens to those of PGE_2 (Hedqvist, 1973a), thus showing a more conclusively presynaptic effect in this preparation than was observed in the cat spleen. As in the spleen, the inhibitory effect of each prostaglandin was antagonised by increasing the external calcium concentration. In addition, it was demonstrated by Stjärne (1973a) that the secretion of NA becomes very sensitive to the inhibitory effect of PGE_2 at low calcium (0.55 to 1.10 mM). It was suggested that PGE_2 may act by depressing the affinity of the secretory mechanism for calcium ions and that this action becomes progressively stronger with falling calcium concentration (Stjärne, 1973c).

An inhibitory effect of PGE_2 on adrenergic transmitter release has been further confirmed in the isolated rabbit heart (Langendorff preparation), where concentrations of 3×10^{-9} to 1.5×10^{-6}M markedly reduced the overflow of NA (fluorimetrically determined) and distinctly counteracted the increase in heart rate to sympathetic nerve stimulation (Hedqvist, Stjärne and Wennmalm, 1970; Hedqvist and Wennmalm, 1971). In this preparation PGE_1 showed similar inhibitory effects at the above dose levels (Wennmalm and Hedqvist, 1971; Hedqvist and Wennmalm, 1971).

The experimental results in this section support the hypothesis that PGE_2 is involved in the control of adrenergic neurosecretion. Since this evidence is backed up by results in several other tissues and by the release of prostaglandins observed in experiments of Section 1, it is tempting to infer that the prostaglandin-feedback mechanism exists as a general phenomenon in all adrenergic neurosecretory processes. This inference is impeded, unfortunately, by an exception to the rule. Although PG's E_1 and E_2 produce a dose-dependent inhibition of lipolysis induced by sympathetic NS in canine subcutaneous adipose tissue (Fredholm and Rosell, 1970), and PGE's are released from white adipose tissue during stimulation of lipolysis in the rat (Shaw and Ramwell, 1968; Christ and Nugteren, 1970), the effect of PGE_2 on transmitter overflow in response to sympathetic NS is variable. PGE_2 seems to inhibit lipolysis without markedly affecting adrenergic transmitter release, suggesting that it acts predominantly at a post-junctional level (Fredholm and Hedqvist, 1973). This is further documented by the fact

that PGE_1 inhibits the lipolytic action of noradrenaline and adrenaline (Steinberg, Vaughan, Nestel and Bergström, 1963).

(iii) The effect of frequency on the PG mechanism

In studies of the isolated rabbit heart (Hedqvist, Stjärne and Wennmalm, 1970), the overflow of NA resulting from stimulation of the sympathetic nerves at frequencies of 1, 5 and 10 Hz was determined fluorimetrically. Measurable quantities of NA were obtained only at frequencies of 5 and 10 Hz. PGE_2 (8×10^{-8}M) caused a marked reduction in the overflow at both frequencies although there was a greater percentage decrease in NA at the lower frequency of 5 Hz. This is not surprising since the rate of release of NA is considerably less at the lower frequency and might conceivably require less PG to restrict its release by say 50%, than would be required to restrict release at 10 Hz by 50%. The results were based upon the use of only one dose-level of PGE_2, however, which limits extrapolation of such a theory. The experiments in this section do show, however, that the transmitter release mechanism becomes less sensitive to increasing doses of PGE_2 as the frequency of RNS is increased from 2 to 10 Hz. A decrease in sensitivity of the sympathetic transmitter release mechanism to PGE_2 with increasing frequency has also been demonstrated in the rabbit heart (Junstad and Wennmalm, 1973b).

Prostaglandins E_1 and E_2 have been shown to reduce responses of the transmurally-stimulated guinea-pig vas deferens whilst potentiating responses of the organ to

exogenous NA (Baum and Shropshire, 1971). This pointed to a presynaptic site of action in the stimulated organ and the magnitude of inhibition was found to be inversely related to the stimulation frequency employed (5, 10 or 20 Hz). In these experiments the period of stimulation (2 s) rather than the total number of pulses was kept constant. The degree of inhibition of adrenergic transmitter release from the kidney during RNS was found to vary inversely with the frequency of stimulation employed. A comparison of the log dose/inhibition curves for the three stimulation frequencies has shown that the sensitivity of the release mechanism to PGE_2 apparently decreases as the frequency increases.

Some workers have concluded that the length of the impulse train rather than the frequency of stimulation determines the inhibitory effect of PGs in adrenergic neurotransmission. In the isolated guinea-pig vas deferens, PGE_2 (2.5 to 5 ng/ml) has been shown to inhibit contractions elicited by field stimulation whilst potentiating contractile responses to NA (Ambache and Zar, 1970, 1971). In agreement with Baum and Shropshire (1971) this indicated that the inhibitory effect was probably prejunctional. This effect was greater when trains of 5 to 10 pulses were applied to the preparation at 50 Hz, than when 50 pulses were applied at 50 Hz. This is not entirely unexpected since the longer pulse train at

such a high frequency presumably causes a considerably higher release of NA than does the short train and probably requires a greater concentration of PG to control this release. The use of such an unphysiological high stimulation frequency unfortunately sheds doubt on the interpretation of these results. They do gain support, however, from experiments carried out by other workers (Illés, Hadhazy, Torma, Vizi and Knoll, 1973) on the isolated guinea-pig vas deferens and atria and the rabbit jejunum. These workers found that the higher the number of pulses applied at a given frequency, the smaller the inhibition by PGE_1 of neuroeffector transmission. PGE_1 also produced a frequency-dependent inhibition of sympathetic NS in the rabbit jejunum (3-15 Hz).

In conclusion, it would appear that the inhibitory effect of prostaglandins E_1 and E_2 on adrenergic neurotransmission is dependent upon both the frequency of nerve or field stimulation and the total number of pulses applied. The reduction in this effect at higher frequencies or higher pulse number may result from (a) a reduction in the sensitivity of the release mechanism (b) an insufficient release of PG relative to NA (c) a combination of (a) and (b) (Junstad and Wennmalm, 1973b). Mechanism (a) seems probable in view of the present results and also those of Baum and Shropshire (1971). With reference to mechanism (b), the release of endogenous prostaglandin during sympathetic nerve stimulation of the dog kidney and the rabbit heart shows no apparent linear correlation with either the frequency or

the transmitter overflow (Dunham and Zimmerman, 1970; Junstad and Wennmalm, 1973b). Thus an increase in stimulation frequency from 2 to 10 Hz only approximately doubled the efflux of prostaglandin E_2-like material per unit time whilst the transmitter overflow increased tenfold. It would seem, therefore, that the endogenous transmitter braking-mechanism is limited by both (a) and (b), leading to suggested mechanism (c).

(iv) Presynaptic effects of prostaglandins A_2 and $F_{2\alpha}$

There are numerous reports in the literature regarding the influence of PGs A_2 and $F_{2\alpha}$ on vascular or contractile responses to sympathetic stimuli (see Discussion, Section 2), although virtually none has described the presynaptic effects of these prostaglandins on adrenergic transmitter release. As a result of the potentiating effects of $PGF_{2\alpha}$ on most adrenergic systems (see Brody and Kadowitz, 1974, for references), it has been suggested that it probably facilitates the release of noradrenaline from nerve endings. The present results have shown that $PGF_{2\alpha}$ does not potentiate transmitter overflow from the kidney in spite of its apparent potentiation of vascular responses to RNS. In addition, ten times as much $PGF_{2\alpha}$ was required to produce overflow inhibition as was required of PGE_2. Hedqvist and Wennmalm (1971) demonstrated that $PGF_{2\alpha}$ was ineffective in producing inhibition of transmitter overflow from the rabbit heart in response to sympathetic NS when compared with identical doses of PGE_2. Malik and McGiff (1975) have shown that the vasoconstrictor effect of $PGF_{2\alpha}$ in the

rat and rabbit kidney is not due to release of catecholamines from sympathetic fibres since chemical sympathectomy did not alter these vasoconstrictor actions. It may be, therefore, that an effect on transmitter release can be induced by sufficiently high concentrations of $PGF_{2\alpha}$, although its primary effect, as evidenced by consistently potentiated vascular responses, is not presynaptic but rather at the postjunctional effector cell.

The requirement of high doses of PGA_2 in the present experiments to inhibit transmitter overflow is probably also an indication that its primary site of action is postjunctional. There have been no other transmitter studies reported to date but many experiments, unlike the present results, have shown a direct vasodilator action of PGA_1 and PGA_2 as well as an ability to reduce vascular responsiveness to NA and nervous stimulation (Horton and Jones, 1969; Kadowitz et al., 1971c; Malik and McGiff, 1975). Only in the rat kidney has PGA_2 been shown to constrict the vascular bed and facilitate adrenergic responses (Malik and McGiff, 1975). Although the presynaptic inhibition of NA release in the present experiments by PGA_2 is in accordance with its vasodilator action in the rabbit kidney, as demonstrated by these last authors, the induction of such an effect requires a high concentration of PGA_2 (10 times that of PGE_2).

It is concluded that, unlike PGE_2, PGs A_2 and $F_{2\alpha}$ exert their influence on adrenergic neurotransmission primarily at a postjunctional level.

SECTION 4

The effect on adrenergic neurotransmission of altering endogenous prostaglandin synthesis in the kidney

Experiments in the previous two sections have demonstrated that intra-arterial administration of prostaglandins to the kidney produces profound effects on the renal vasculature and on its responsiveness to adrenergic stimuli. In various attempts to assess the importance of endogenous prostaglandins in autoregulatory function and control of blood flow distribution in the kidney, the technique of reducing or abolishing endogenous prostaglandin biosynthesis has been employed (Herbaczynska-Cedro and Vane, 1973; Itskovitz, Terragno and McGiff, 1974; Needleman, Marshall and Johnson, 1974b). This method of study affords several advantages over that of exogenous prostaglandin administration (see introduction to Part 4a) but has the disadvantage of producing inhibition of all prostaglandin synthesis rather than inhibition of a particular prostaglandin. An alternative method of altering endogenous synthesis is to stimulate prostaglandin synthesis by administration of precursor acid (see introduction to Part 4b for references). In this way the type of prostaglandin formed is predetermined according to which precursor acid is used.

This section explores the use of both techniques.

Part 4a: Inhibition of endogenous prostaglandin synthesis in the kidney and subsequent effects on adrenergic neurotransmission

The discovery by Vane and his colleagues (Vane, 1971; Ferreira, Moncada and Vane, 1971; Smith and Willis, 1971), that aspirin-like drugs reversibly inhibit or abolish prostaglandin biosynthesis, has provided workers in the field of prostaglandin research with an invaluable pharmacological tool. In assessing the possible physiological role of a naturally occurring body substance, it is clearly advantageous to be able to examine the effect on body function of eliminating that substance. The following experiments were designed in the hope of strengthening the hypothesis that endogenous prostaglandins have a role in the control of adrenergic transmitter release. The two non-steroidal anti-inflammatory compounds, indomethacin and meclofenamate have been found to be even more potent than aspirin in their ability to inhibit prostaglandin biosynthesis (Flower, Gryglewski, Herbaczynska-Cedro and Vane, 1972). For this reason they were used in preference to aspirin in the present experiments.

Experiment 1: Effects of indomethacin and sodium meclofenamate on adrenergic neurotransmission in the isolated rabbit kidney

Methods

Experiments were performed on eighteen isolated kidneys which were dissected and set up for perfusion with

Dextran-Tyrode solution as described in Section 2. Transmitter stores were labelled by infusion of 25 μCi ^{3}H-NA and transmitter overflow was monitored by 1 min fractionation of the perfusate for radioactive counting, as described in detail in Section 3. The renal nerve was placed on platinium electrodes and stimulated with a Grass S5 stimulator delivering 30 s trains of pulses (5 Hz, 2 ms, 7-15 V).

Solutions of indomethacin (Merck, Sharpe and Dohme) and sodium meclofenamate (Parke Davies) were made up freshly immediately prior to use. Each solution was made up by dissolving about 10 mg drug in approximately 0.5 ml pure ethanol.
Solubility was maintained by adjusting the pH to 8 using 0.2 ml pH8 Tris buffer before final dilution with 0.9% saline to a concentration of 1 mg/ml.
Vascular responses to nerve stimulation were obtained by stimulating the renal nerve as above for 30 second periods at 10 min intervals. Vascular responses to exogenous noradrenaline were obtained by infusion over 30 seconds of a suitable dose of noradrenaline, usually 0.25 - 1.0 μg total.

Results a) Indomethacin

Infusion of indomethacin at a concentration of 4.4×10^{-5}M into the isolated kidney sometimes caused a slight increase in the resting perfusion pressure (up to 5 mm Hg) and either no effect or a slight decrease in the pressor

responses resulting from RNS. The slight inhibitory effect on effector responses to RNS did not reflect a corresponding decrease in transmitter overflow, however. On the contrary, the overflow of tritium during RNS increased significantly about 8 min after the start of an infusion of indomethacin and plateaued after about 15 min of infusion. The average increase of ^{3}H-NA overflow in response to RNS was 41.5 ± 7% (Mean ± S.E.M.) in thirteen experiments. This value was significant at the $p < 0.001$ level, according to Student's t-test for paired variates. After ending the infusion, the transmitter overflow fell off approaching the pre-infusion level within ten to twenty minutes (Fig. 4a). However, in some experiments the transmitter overflow remained at a slightly elevated level throughout post-infusion controls. Indomethacin did not affect resting transmitter overflow (Fig. 4a).

In view of its potentiating effect on transmitter overflow, the effect of indomethacin on pressor responses to RNS was surprising. In order to investivate whether this inhibitory effect was primarily postjunctional, the effect of indomethacin on pressor responses to NA was examined. Infusion of indomethacin as above also produced inhibition of pressor responses to NA (Fig. 4b) thus confirming a postjunctional site of action.

Results b) Sodium meclofenamate

In three experiments, sodium meclofenamate was infused in place of indomethacin, also at a concentration of 5×10^{-5}M. Its effect on resting perfusion pressure was

Figure 4a: Isolated, perfused rabbit kidney. Effect of indomethacin on tritium overflow (^{3}H-overflow) resulting from renal nerve stimulation (NS).

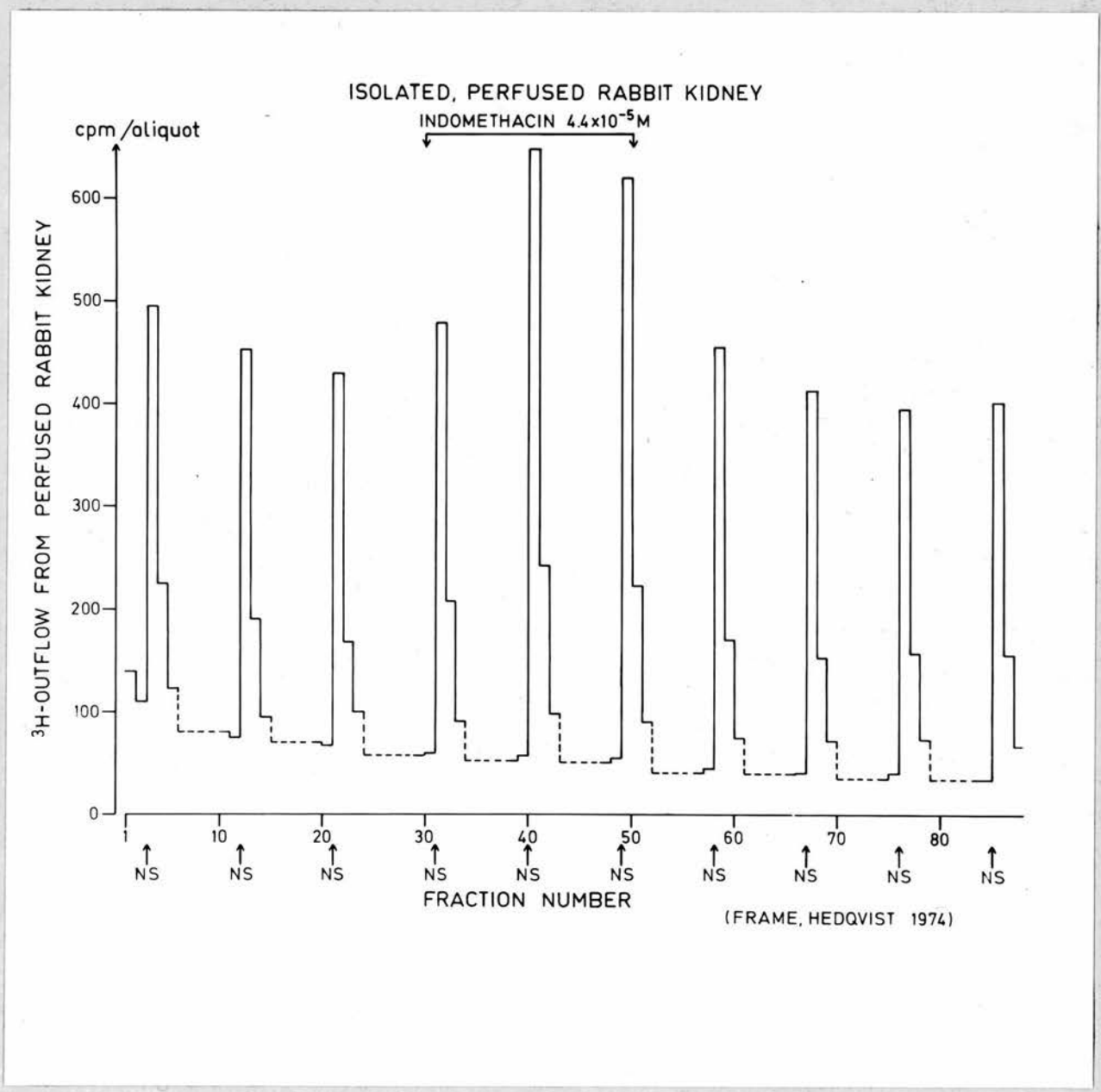
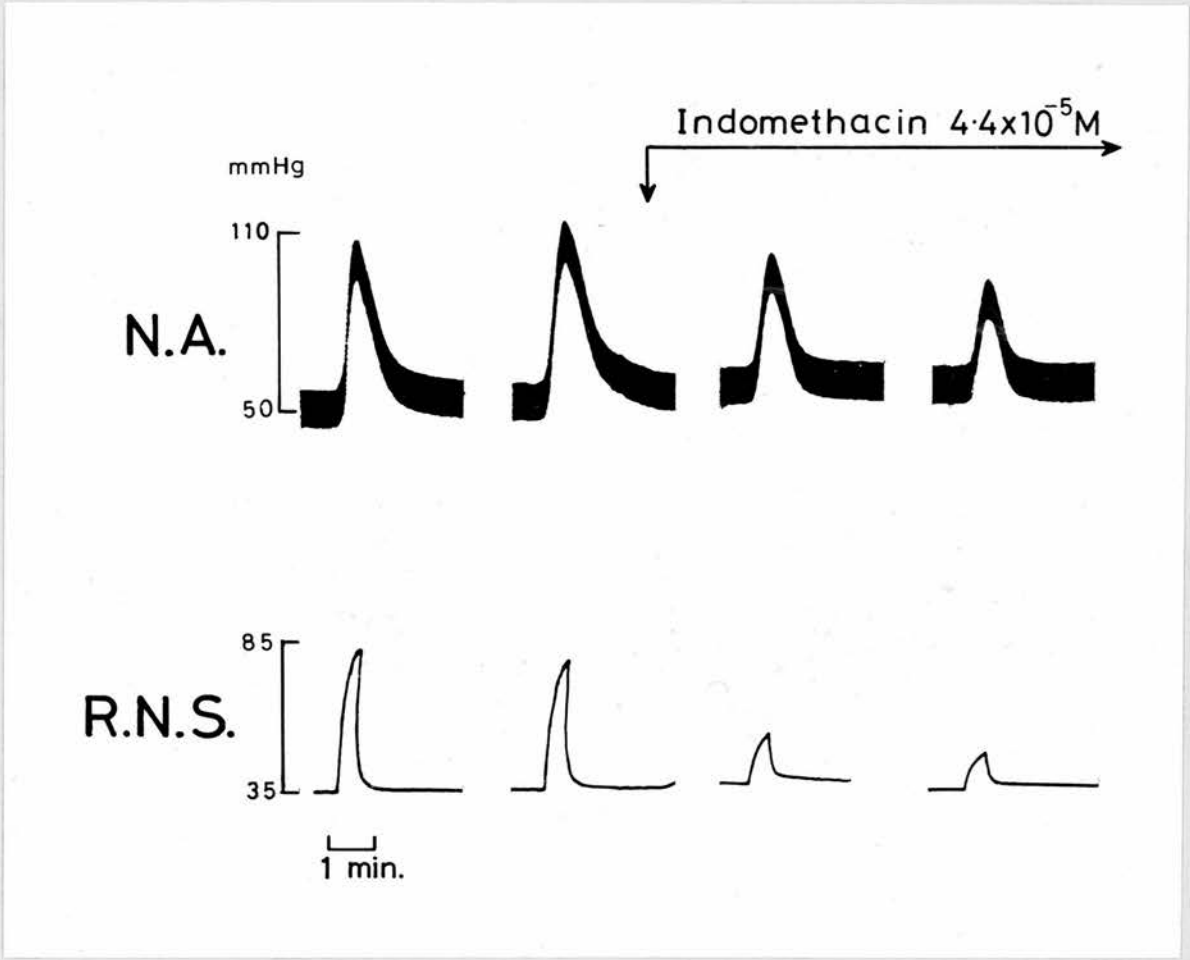


Figure 4b: Isolated, perfused rabbit kidney. Effect of indomethacin on vascular responses to renal nerve stimulation (RNS) and noradrenaline (NA).



negligible although it caused a slight inhibition of effector responses to RNS. Its effect on transmitter overflow was qualitatively similar to that of indomethacin. It did not affect resting transmitter overflow but increased overflow resulting from RNS. The average increase was 23.3 ± 3.5% which was significant at the $p < 0.05$ level according to Student's t-test for paired variates. This drug, therefore, was less potent than indomethacin on an equimolar basis.

Conclusion

It may be concluded from these experiments that prostaglandin synthesis inhibitors induce a marked elevation of transmitter release from adrenergic nerve endings in the kidney. This observation is to be expected if an endogenous braking-mechanism has been partially or wholly removed. The evidence, therefore, supports the view that endogenous prostaglandins do exert a negative-feedback control over adrenergic transmitter release. The question now arises as to whether the potency of exogenous PGE as an inhibitor of transmitter release is increased when the competition of endogenous prostaglandin is removed by synthesis inhibition. The following experiment was performed to examine the phenomenon of "endogenous PG" versus "exogenous PG" in the control of transmitter release.

Experiment 2: Effect of PGE_2 on transmitter overflow in the presence of indomethacin

Methods

This experiment was performed on three isolated Dextran-Tyrode perfused kidneys. The renal nerve was stimulated using parameters identical to those in Experiment 1 (5 Hz, 2 ms, 7-15 V for 30 s) at 10 min intervals, the kidney having been labelled with 25 μCi ^{3}H-NA. The perfusate was fractionated as in Experiment 1 for radioactive counting. After three control stimulation periods, indomethacin was infused as previously at a concentration of 4.4×10^{-5}M. Two stimulation periods were carried out with indomethacin alone, thus allowing a plateau of its activity to be reached. PGE_2 was then infused at a concentration of 4.5×10^{-7}M for two minutes prior to and until the end of the next stimulation period. At the end of this stimulation period, infusion of both indomethacin and PGE_2 was ended. Two or three further post-test control stimulations were collected.

Results

During indomethacin treatment, as described in methods, transmitter overflow increased to a plateau level before infusion of PGE_2. PGE_2, infused during indomethacin treatment, was still effective in producing an inhibition of ^{3}H-NA overflow in response to RNS. The average inhibition produced by a concentration of 4.5×10^{-7}M PGE_2 was 61.3 ± 4.6% (mean ± S.E.M., n = 3). This mean value was found to be significantly greater ($p < 0.02$) than the inhibition of

39.6 ± 4.5% (n = 5) produced by PGE_2 of the same concentration in the absence of indomethacin, according to the Student's t-test for unpaired variates (See Table 4).

Conclusion

The primary effect of indomethacin, to increase the transmitter overflow resulting from RNS, was again observed in these experiments. Moreover, a given dose of PGE_2 showed an increased ability to inhibit transmitter overflow during indomethacin treatment. This increased potency of exogenous PGE_2 may be attributable to removal of a local braking mechanism, assuming that administered PGE_2 normally has to compete with endogenous PG for active sites in the nerve terminal.

Discussion

In view of the cumulative evidence in the previous sections suggesting that PGE_2 is the major intrarenal prostaglandin (see also General Introduction), abolition of prostaglandin synthesis by indomethacin or meclofenamate would be expected to produce intrarenal effects opposite to those incurred by PGE_2 administration. These drugs might be expected, therefore, to

i) increase basal renal vascular resistance

ii) increase responsiveness of renal vasculature to circulating and locally-formed NA.

iii) increase the release of NA from adrenergic nerve terminals.

The results obtained in this section are discussed with reference to each of the above proposed modes of action.

(i) Effect on basal renal vascular resistance

The use of the isolated kidney preparation afforded the opportunity of examining the direct action of indomethacin on renal vasculature, since the possible intervention of systemic reflexes and/or circulating catecholamines was eliminated.

If endogenous prostaglandins are responsible for maintenance of resting renal blood flow, in part, by a direct vasodilator action on renal vasculature, their removal would be expected to result in an increased vascular resistance and a resultant reduction in renal blood flow through the isolated organ. In the current experiments, indomethacin infusion (15.8 µg/ml) did produce a slight increase (ca. 5 mm Hg) in resting perfusion pressure, thus reflecting an increase in vascular resistance, flow being kept constant. Further evidence in support of mode of action (i) has been provided by Malik and McGiff (1975), also in the isolated rabbit kidney. These workers found that doses of indomethacin from 0.1 - 1.0 µg/ml had no effect on resting perfusion pressure whilst 5 µg/ml caused an increase in perfusion pressure of about 5.5 mm Hg. Similarly, Lonigro, Itskovitz, Crowshaw and McGiff (1973b) showed that application of indomethacin or meclofenamate (concentrations not stated) to the blood-perfused isolated dog kidney decreased the basal concentration of PGE-like substance in the renal venous blood and caused a reduction in renal blood flow in face of constant perfusion pressure. This was confirmed by them in the in situ dog kidney. They

carried out a parallel study on femoral blood flow and showed that it was variably and insignificantly affected by indomethacin or meclofenamate, suggesting some selectivity of action of these drugs in different vascular beds. Selectivity is further indicated by the results of Fredholm and Hedqvist (1975) who have shown that indomethacin (5 μg/ml) has no effect on blood flow through perfused canine subcutaneous adipose tissue, *in situ.* Ferreira, Moncada and Vane (1973) express some doubt as to the validity of *isolated* organ studies, as they demonstrated in the isolated spleen that indomethacin caused an increase in resting perfusion pressure only after 2-3 hours of perfusion. (There was usually an increase in basal output of prostaglandins at this time in the absence of indomethacin.) If indomethacin was infused in concentrations ranging from 0.3 - 5 μg/ml about 30-60 min from the start of the perfusion, no such increase in basal perfusion pressure was observed. The increased basal output of prostaglandins after 2-3 hours may be attributable to tissue damage, a condition which is known to release prostaglandins (Piper and Vane, 1971). The experiments described in this section did not include analysis of perfusate prostaglandin content but since kidneys were perfused for more than an hour before indomethacin was infused, the basal output may have been higher than normal. An apparently selective action of indomethacin on inner cortical vasculature has been observed, however, in the isolated dog kidney by Itskovitz *et al.* (1974), where the effect of prostaglandin on the distribution of renal blood

flow was examined. A bolus injection of 2-5 mg indomethacin into the blood-perfused isolated canine kidney not only reduced total renal blood flow but, in particular, reduced inner cortical flow. Interestingly, infusion of PGE_2 up to a blood concentration of 2 μg/ml did not overcome the reduction in renal blood flow induced by indomethacin. The effect of indomethacin was reduced by 54%, however. The most important observation was that the remaining effect of indomethacin was accounted for almost entirely by a decrease in the inner cortical component of blood flow. Since PGE_2 has been shown by Larsson and Änggård (1974) to increase juxtamedullary or inner cortical blood flow, indomethacin's action seems to result specifically from a block in prostaglandin synthesis. The inability of PGE_2 to reverse the effect of indomethacin completely in the experiments of Itskovitz et al. (1974) is possibly attributable to its partial degradation in the renal cortex en route before reaching its endogenous site of action.

In view of the various results discussed so far, it seems probable that the action of indomethacin on basal vascular resistance is dependent upon the degree of direct control by prostaglandin at that time. Although a non-prostaglandin-mediated action of indomethacin may exist in the kidney, present results and the work of Itskovitz et al. (1974) infer that indomethacin produces its vasoconstrictor effect on renal vasculature by inhibiting prostaglandin synthesis.

(ii) Effect on vascular responsiveness to circulating and locally-formed NA

If an inhibitory effect on vascular responsiveness to catecholamines constitutes a part of the action of endogenous prostaglandins in the kidney, inhibition of their synthesis would be expected to augment the vasoconstrictor action of catecholamines. The results in this section showed slight augmentation of responses to RNS at a concentration of 1.8×10^{-5}M indomethacin, but at a concentration of 4.4×10^{-5}M and 5×10^{-5}M respectively, both indomethacin and meclofenamate depressed vasoconstrictor responses to RNS and NA. Malik and McGiff (1975), in a similar study of the rabbit kidney, showed that indomethacin or meclofenamate at concentrations from 0.1 - 1.0 µg/ml augmented vasoconstrictor responses to RNS but had no effect on equiconstrictor responses to NA. This suggested a primary prejunctional role for endogenous prostaglandin in the kidney. Needleman et al. (1974b) on the other hand showed potentiation of responses to both RNS and NA in the isolated rat kidney by 2.5 µg/ml indomethacin. These results are in direct conflict with those obtained by Malik and McGiff (1974, 1975) in the isolated rat kidney, where they found that 1 µg/ml indomethacin or meclofenamate reduced to a similar degree the vasoconstrictor responses to RNS and NA. The last authors used Tyrode perfusion at 6 ml/min whilst Needleman et al. used Krebs at 4 ml/min, but such differences are unlikely to account for the discrepancy in results. The stimulation frequencies employed varied from 2 Hz (Malik and McGiff) to 3-6 Hz (Needleman et al.). Although small, these differences

may be important in view of the frequency-dependent effects of indomethacin described later in this discussion. Malik and McGiff have even suggested that the rat kidney is unsuitable for the study of prostaglandins as antihypertensive agents, since PGE_2 and PGA_2 may be prohypertensive there. To compare, therefore, only the results obtained with the rabbit kidney, the discrepancy between present results and those of Malik and McGiff is almost certainly attributable to the difference in the dose levels of indomethacin utilised.

Aiken and Vane (1973) provide supportive evidence for the hypothesis that intrarenal PGE_2 generation attenuates increases in vascular resistance in the dog kidney. They found that indomethacin, 0.1 - 2 mg/Kg, or meclofenamate, 1 mg/Kg, given intravenously, augmented renal vasoconstrictor responses to NA.

Some interesting experiments have been conducted on isolated blood vessel preparations. Gryglewski and Korbut (1975) have demonstrated that application of indomethacin, 3 µg/ml, to the perfused rabbit ear artery <u>did not</u> potentiate vasoconstrictor responses to NA but did effect an increased tolerance to NA. De la Lande, Hall, Kennedy and Higgins (1975) also examined the effect of indomethacin (3 µg/ml) on responses of the rabbit ear artery, this time to NS. They found that it did not enhance responses to NS as would have been expected if PGE_2 were released physiologically during stimulation to depress the responses. Jackson and Hall (1973), on the other hand, found that aspirin, 200 µg/ml <u>did</u> potentiate responses to the rabbit ear artery to intraluminal NA <u>and</u> to electrical stimulation. Cocaine partially

inhibited these effects of aspirin and it is suggested that the latter may act by a similar extraneuronal mechanism to cocaine, preventing reuptake of NA. This seems feasible since if aspirin's only action were to prevent the braking effect of PGE_2 on transmitter release, one would expect cocaine to potentiate its action by preventing the reuptake of the already-elevated transmitter released into the synaptic cleft, hence producing a further augmentation of the response to NS. Caution must be exercised, however, in interpreting the results of experiments in which such high dose-levels were employed. Certain effects of the aspirin may have been non-specific.

The importance of species in the study of prostaglandin-indomethacin-catecholamine interactions is further highlighted by a recent study in the isolated rat arteriole (Horrobin, Manku, Karmali, Nassar and Davies, 1974). These workers showed that aspirin, 5 - 20 μg/ml, or indomethacin, 5 μg/ml, could reversibly depress the responsiveness of the arteriole to NA. Furthermore, PGE_2 at a concentration of 1 pg/ml restored responsiveness of the arteriole to NA. The depression by aspirin or indomethacin of vascular responsiveness was not specific to alpha receptors, however, but also occurred when vasoconstriction was induced with angiotensin and vasopressin. This points to a more generalised action of indomethacin, perhaps on excitation-contraction coupling. The observations nevertheless suggest that the effect results from a block in prostaglandin synthesis.

Greenberg (1974, 1975) has investigated the possibility that stimulus frequency and train length may influence the

action of indomethacin on vasoconstrictor responses of the rabbit portal vein to electrical stimulation. He discovered that indomethacin (10 μg/ml) caused significant potentiation of responses to 2 Hz field stimulation at all train lengths, but significantly reduced the response to 5 Hz at all train lengths. Inhibition of noradrenaline synthesis with α-methyl-p-tyrosine abolished indomethacin's potentiating effect. It is suggested that the noradrenaline released at low frequencies is newly synthesised and that its release is controlled by endogenous prostaglandins. These results are in accordance with the frequency study described in Section 3, where PGE_2 produced its greatest inhibitory effect on transmitter overflow at the lowest frequency of 2 Hz. Greenberg does not proffer any explanation of the inhibitory effect of indomethacin observed at a frequency of 5 Hz. It would be interesting to observe whether in this preparation, as in that used by Malik and McGiff, a dose of less than 5 μg/ml indomethacin would produce a potentiating rather than an inhibitory effect on NS responses.

In conclusion, it seems that the effects of indomethacin on vascular responsiveness are variable according to the dose-level utilised, the animal species and, for effects on responses to electrical stimulation, the frequency of stimulation.

(iii) Effect on transmitter release from adrenergic nerve terminals

There have been relatively few studies to date in which the action of the anti-inflammatory drugs on sympathetic

transmitter release has been examined. Junstad and Wennmalm (1972) demonstrated an increase in renal excretion of NA in rats after treatment with indomethacin (0.5 - 2.0 mg/Kg). Since the renal excretion of NA apparently reflects the release of transmitter from adrenergic nerves (Leduc, 1961; Stjärne, 1971), these results provide substantial evidence in favour of prostaglandin-mediated control of adrenergic transmitter release. In further studies with indomethacin, Chanh, Junstad and Wennmalm (1972) found that, in doses of ca. 16 µg/ml, it inhibited outflow of prostaglandin and increased the release of NA from the isolated rabbit heart during sympathetic NS. Indomethacin did not alter the release of NA during resting periods, however, nor did it affect reuptake of exogenous NA. The authors concluded that indomethacin causes 'dis-inhibition' of NA release. Fredholm and Hedqvist (1973a) have confirmed this effect in the transmurally-stimulated guinea pig vas deferens. They showed that indomethacin (2-10 µg/ml) did not affect resting radioactive transmitter efflux but did increase the overflow of ^{3}H-NA in response to transmural stimulation. The duration of the effect was similar to that described in this section on the isolated kidney. The same authors (1975) have demonstrated that indomethacin (5 µg/ml blood) was without effect on ^{3}H-NA overflow before, during and after NS at 4 Hz in perfused canine subcutaneous adipose tissue. This was in agreement with their previous findings (1973) that PGE_2 had no consistent effects on vasoconstriction or ^{3}H-NA overflow induced by NS in that bed. The lack of effect of indomethacin on blood flow, lipolysis

or ^{3}H-NA overflow throughout NS induced the conclusion that endogenous prostaglandins are of minor importance as feedback inhibitors of lipolysis in adipose tissue. These workers emphasise, however, that conclusions as to the role of endogenous prostaglandins in tissues cannot be drawn solely from results obtained by inhibition of synthesis using the anti-inflammatory drugs. Indomethacin, for example, does produce some biological effects which are quite unrelated to inhibition of prostaglandin synthesis (Fredholm and Hedqvist, 1975). The effect of indomethacin on adrenergic transmitter release in this section, however, does apparently relate to an inhibition of endogenous prostaglandin synthesis, since PGE_2 is capable of opposing and overcoming that effect.

Part 4b: Stimulation of endogenous prostaglandin synthesis in the kidney and subsequent effects on adrenergic neurotransmission

It is now well-established that prostaglandins are not synthesised and stored in tissues prior to their release (Ramwell et al., 1966; Davies et al., 1968; Piper and Vane, 1971). Synthesis occurs spontaneously in response to various stimuli (see Introduction) and is followed by an immediate release of the newly-formed prostaglandin. In addition, prostaglandins of the E and F series are rapidly metabolised on circulation through the lungs (Ferreira and Vane, 1967; Vane, 1969). It has been concluded by Vane (1969) that this is an efficient mechanism by which prostaglandins are prevented from reaching the arterial circulation. These observations indicate that prostaglandins are probably not circulating hormones, with the possible exception of PGA_2, which has been shown to escape degradation by the lungs (Horton and Jones, 1969; McGiff et al., 1969). They are rather formed and act locally. It may, therefore, be regarded as unphysiological to investigate the effects of prostaglandins on an organ by intraarterial administration. Furthermore, the prostaglandin may not reach its main site of action as a result of metabolism en route. This is particularly relevant in the kidney which possesses a high amount of the prostaglandin-degrading enzyme, prostaglandin dehydrogenase, in its cortex (Larsson and Änggård, 1973a). Thus a more physiological approach to the study of endogenous prostaglandin

function would seem to be local stimulation of prostaglandin synthesis. It has been shown that the rate-limiting factor in the synthesis of prostaglandins is the availability of precursor acid (Samuelsson, 1970). Consequently, the synthesis of endogenous prostaglandin may be increased or "stimulated" by administration of exogenous precursor (Larsson and Änggård, 1973b, 1974).

Experiment 1: Stimulation of endogenous PG synthesis in the isolated kidney using the precursor, arachidonic acid and subsequent effects of this on adrenergic neurotransmission

As indicated in the introduction to this section, prostaglandin biosynthesis may be evoked by administration of exogenous precursor acid. Since the major renal prostaglandins have been conclusively identified as PGE_2 and $PGF_{2\alpha}$ (possibly some PGA_2)(see Davis and Horton, 1972 for references), their precursor, arachidonic acid, was used in the present experiments. The primary aim of the experiments was to assess the accessibility of newly-formed prostaglandins to cortical nerve endings.

Methods

Preparation of arachidonic acid

Arachidonic acid (Sigma Chemicals, Grade 1, 99% pure, porcine liver) was purified and made into the sodium salt shortly before use, as described by Larsson and Änggård (1974). It was hoped that purification by silicic acid column chromstography would eliminate prostaglandin contamination, if any, in the 1% impurity.

Columns (approximately 15 x 1 cm) were made up with 0.75 g silicic acid (Unisil, 100-200 mesh, Clarkson Chemical Co) in hexane. Arachidonic acid (10 mg) was applied to the column in 0.5 ml hexane. The column was then washed with 20 ml hexane to remove non-polar lipids. This was followed by elution of arachidonic acid from the column with 20 ml 5% ether in hexane. A recovery experiment with labelled arachidonic acid showed that 84% of it was recovered in the ether-hexane fraction and a further 2% in the hexane fraction. The recovery was assumed identical for other columns made with the same batch of Unisil.

The purified arachidonic acid was then converted to the anion by brief treatment with 0.5 N KOH in pure ethanol. The ethanol was evaporated under nitrogen and the arachidonate dissolved in 0.9% saline. Ascorbic acid, 50 µg/ml was added as an anti-oxidant. This solution was stored deep-frozen under nitrogen for a maximum of 48 hours, at the advice of Larsson (personal communication). The stock solution was diluted as required with 0.9% saline.

Experimental procedure

Thirteen rabbit kidneys (from the left side) were set up for isolated Dextran-Tyrode perfusion and perfusate collection as previously described in Sections 2 and 3. Each kidney was labelled with 25 µCi ^{3}H-NA for transmitter studies. Parameters for stimulation of the renal nerve were chosen midway in the range of frequencies previously studied, namely 5 Hz for 30 s (2 ms duration, 7-15 V). The period of infusion of arachidonate was extended to approximately twice

that used for prostaglandin infusion prior to RNS to allow time for the former to reach the site of endogenous prostaglandin synthesis. Thus arachidonate infusion was begun 5 min prior to RNS and continued throughout RNS. In cumulative dose experiments, each concentration of arachidonate was infused as just described, to successive stimulations of the renal nerve. Infusion concentrations ranged from 10^{-7} to 5.2 x 10^{-5}M. A minimum of three nerve stimulation periods were monitored for transmitter overflow prior to arachidonate application.

Results

In the first experiment, infusion of the lowest concentration of arachidonate, 10^{-7}M, produced neither inhibition of transmitter overflow nor inhibition of vascular responses resulting from RNS. At an infusion concentration of 10^{-6}M, arachidonate again produced no inhibition of transmitter overflow although there was slight inhibition of vascular responses to RNS. When the dose of arachidonate was increased to 2 x 10^{-5}M for 5 min prior to and also during RNS, transmitter overflow was inhibited by 35.3 $\pm$ 4.0% (n = 5. see Table 4).

On addition of cumulative doses of arachidonate, 10^{-5} to 5.2 x 10^{-5}M, a dose-dependent inhibition of transmitter overflow during RNS was observed. (Fig. 4c). This inhibition was accompanied by concomitant inhibition of vascular responses to RNS (Fig. 4d). In most experiments, a slight reduction in perfusion pressure was observed throughout the duration of arachidonate infusion. Partial

Figure 4c: Isolated, perfused rabbit kidney. Effect of cumulative doses of arachidonic acid on tritium overflow (^{3}H-overflow) resulting from renal nerve stimulation (NS).

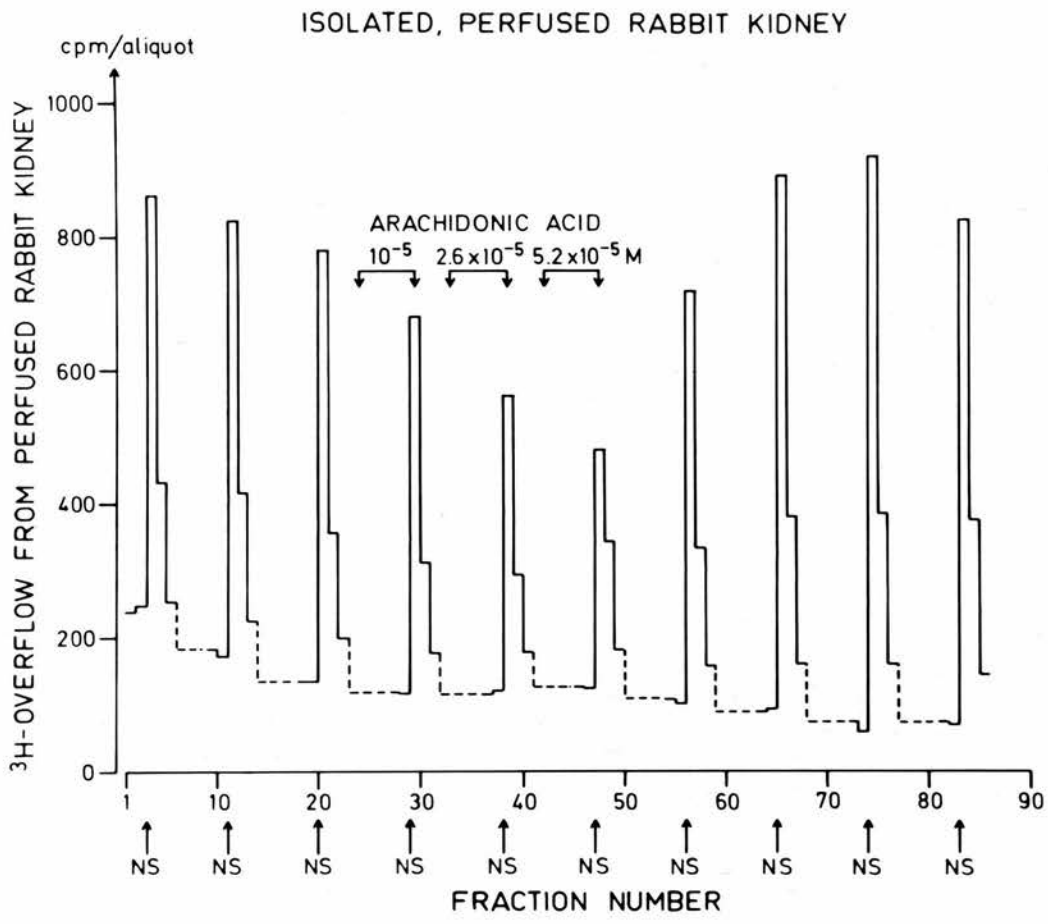
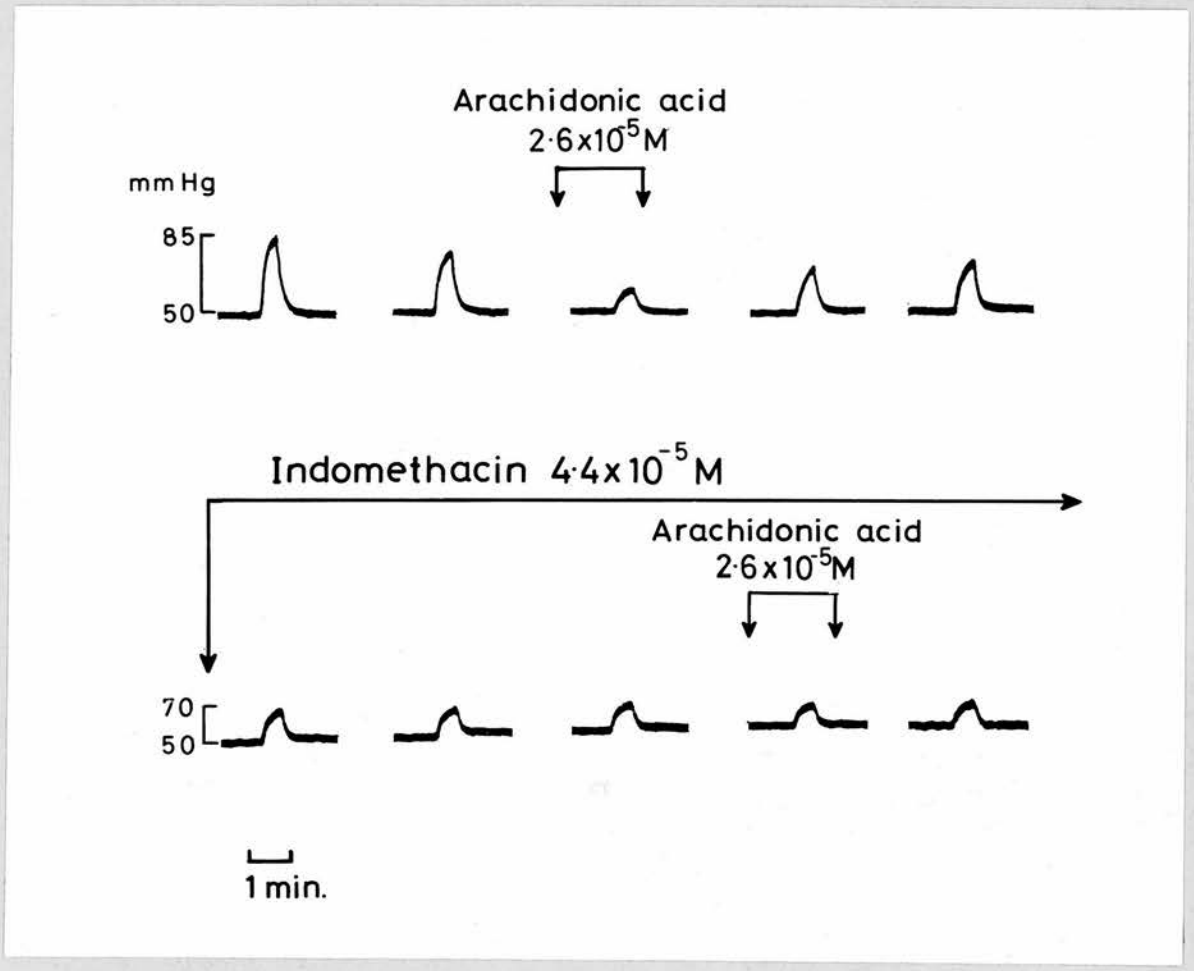


Figure 4d: Isolated, perfused rabbit kidney. Effect of arachidonic acid alone and of arachidonic acid in the presence of indomethacin on vascular responses to renal nerve stimulation.



or complete recovery from inhibition of transmitter overflow usually occurred within 10-20 min of ending the infusion although in some experiments the overflow remained depressed. Recovery of vascular responses was generally slower, occurring about 20-30 min after ending the arachidonate infusion.

Conclusion

These experiments have shown that arachidonate, in concentrations ranging from 10^{-5} to 5.2×10^{-5}M, is capable of mimicking the effects of PGE_2 on transmitter overflow and vascular responses to RNS (Table 4). Since arachidonate is apparently pharmacologically inactive in itself (Dakhil and Vogt, 1962), its action is probably attributable to the spontaneous synthesis of prostaglandin within the kidney. Prostaglandin formation was not monitored either in renal venous effluent or in urine. Consequently, it was necessary to investigate whether the effects of arachidonate could be abolished by inhibiting prostaglandin biosynthesis.

Experiment 2: Inhibition by indomethacin of endogenous prostaglandin biosynthesis from arachidonate and subsequent effects on adrenergic neurotransmission

By administering arachidonate to the isolated kidney in the presence of the PG synthesis inhibitor, indomethacin, it was hoped to find out whether arachidonate produces its actions on neurotransmission per se, or as a result of prostaglandin formation. If resultant from prostaglandin

formation, the actions of arachidonate should be reduced or abolished in the presence of indomethacin.

Methods

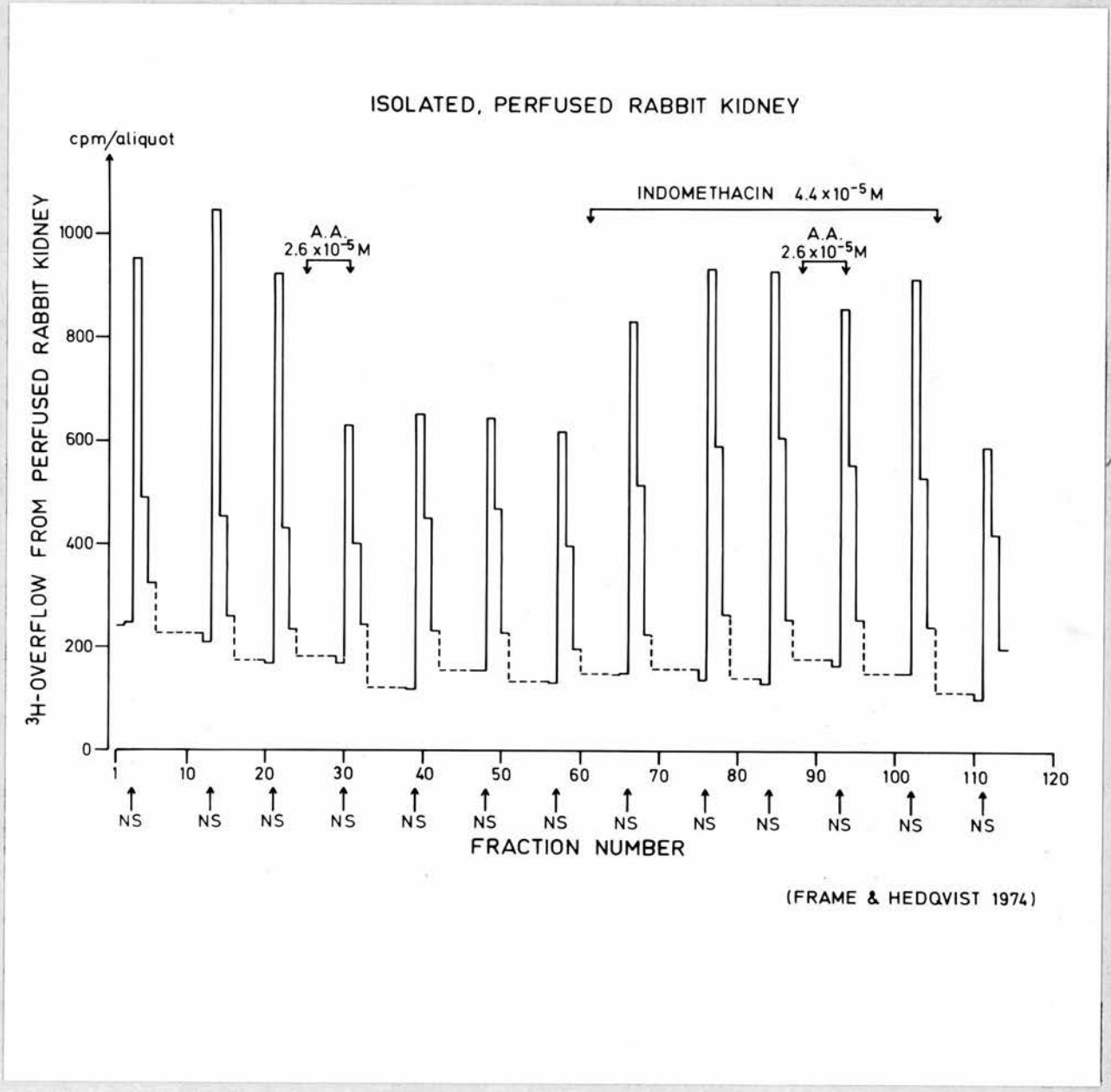
Five rabbit kidneys were set up for isolated perfusion and transmitter collection as described in Experiment 1. Three control responses to RNS were obtained by stimulation to the renal nerve as before at 5 Hz for 30 s. Arachidonate was then infused at a concentration of 2.6×10^{-5}M for 5 min prior to and also during RNS. A further three post-test controls were obtained. Indomethacin, prepared as in Section 4a, was then infused at a concentration of 4.4×10^{-5}M. Three test samples were collected, by which time a plateau of elevated transmitter overflow should be reached. Arachidonate was then infused, exactly as before, in the presence of indomethacin. Infusion of indomethacin was maintained for one post-arachidonate stimulation period and was terminated immediately thereafter. One or more post-indomethacin control stimulations were carried out. An example of the sequence of arachidonate and/or indomethacin administration can be seen in Fig. 4e.

Results

The results are presented in Table 4. Transmitter overflow during arachidonate infusion was calculated as a percentage of the preceding control output. The overflow during arachidonate infusion in the presence of indomethacin was calculated as

$$\frac{\text{overflow during arachidonate + indomethacin}}{\text{previous overflow during indomethacin}} \times 100\%$$

Figure 4e: Isolated, perfused rabbit kidney. Effect of arachidonic acid (AA) alone and of arachidonic acid in the presence of indomethacin on tritium overflow (^{3}H-overflow) resulting from renal nerve stimulation (NS).



The percentage change in overflow during various treatment was calculated by subtraction of the above-calculated values from 100%.

Table 4 Percentage change of transmitter overflow to RNS (5 Hz, 150 pulses) by PGE_2 (4.5×10^{-7}M), arachidonate (2.6×10^{-5}M) and indomethacin (4.4×10^{-5}M), and by PGE_2 and arachidonate in the presence of indomethacin

Administered Compound	Pre-treatment		Difference None–indomethacin
	None	Indomethacin	
PGE_2	$-39.6 \pm 4.5(5)$ $p < 0.001$	$-61.3 \pm 4.6(3)$ $p < 0.01$	21.7 ± 6.4 $p < 0.02$
Arachidonate	$-35.3 \pm 4.0(5)$ $p < 0.001$	$-10.6 \pm 4.4(5)$ $p < 0.1$ (NS)	24.7 ± 5.9 $p < 0.001$
Indomethacin	$+41.5 \pm 7.0(13)$ $p < 0.001$		

Mean values ± S.E.M., figures within brackets = number of experiments. Statistical analysis according to Student's t-test. NS = not significant.

Conclusion

It is apparent from these experiments that indomethacin significantly reduces the biological activity of arachidonate. Thus the inhibitory effect on vascular responses and transmitter overflow was primarily that of newly-formed prostaglandin rather than that of arachidonate itself. A small residual effect of arachidonate during indomethacin treatment

is not unexpected, since indomethacin, in doses similar to that used presently, has been shown to markedly inhibit but not abolish the conversion of arachidonate to PGE_2 in rabbit kidney and in rat kidney homogenates (Larsson and Änggård, 1974; Fredholm, Hedqvist and Larsson, unpublished observations).

Discussion

The probable sites of action of arachidonate are discussed in terms of (i) direct action on renal vasculature, (ii) influence on vascular responsiveness to catecholamines, (iii) influence on adrenergic transmitter release.

(i) Direct action

The use of an isolated organ preparation for this study again eliminates reflex or systemic influences and hence sympathetic tone. Any reduction in perfusion pressure effected by arachidonate may be ascribed, therefore, to a direct action on renal vasculature. The capacity of indomethacin to reduce or abolish arachidonate's action confirms the belief that the latter is mediated by newly-formed prostaglandins. It is curious that in these experiments, arachidonate produced no effect on perfusion pressure at concentrations of 10^{-6}M (300 ng/ml) or less whilst in similar experiments on the isolated kidney, Malik and McGiff (1975) found that concentrations of 100 ng/ml produced a fall in perfusion pressure. The arachidonic acid utilised by them, however, was not subjected to further column chromatography and may have contained an active contaminant. This

possibility cannot be excluded since these authors only showed an average of 57% reduction in the vasodilator action of arachidonic acid during infusion of indomethacin (1 μg/ml). An alternative explanation of the discrepancy is that in present experiments, the concentration of arachidonate was estimated assuming complete salt formation. It is possible, therefore, that the concentration of arachidonate was over-estimated.

Arachidonic acid demonstrates an ability to redistribute renal blood flow in several in vivo kidney experiments. Larsson and Änggård (1974) have shown, using the radioactive microsphere technique, that sodium arachidonate (15-25 μg/kg/min) increases the ratio of juxtamedullary to superficial cortical blood flow in the rabbit kidney. Indomethacin (10-20 mg/kg) was found to abolish this effect. A similar investigation was performed by Chang, Splawinski, Oates and Nies (1975) and their results confirmed this ability of sodium arachidonate (3-10 μg/kg/min) to redistribute intrarenal blood flow in favour of the juxtamedullary cortex, in the dog kidney. The possible intervention of neurogenic or circulating factors in the two fore-mentioned reports constitutes a major drawback to any deduction of arachidonate's mode of action.

(ii) Influence of arachidonate on vascular responsiveness to catecholamines

The ability of arachidonate at a concentration of 10^{-6}M to inhibit vascular responses to NS in present experiments, without apparently altering transmitter overflow, indicates

the existence of a postjunctional site of action. This is not unexpected in view of the direct actions of arachidonate discussed in (i). At dose-levels of 2×10^{-5}M and above, a prejunctional site of action also becomes apparent. The primary site of action may not be postjunctional, however, despite occurrence of the latter prior to any effect on transmitter release. It is possible that arachidonate gains direct access to renal vasculature on infusion and is synthesised there into prostaglandins, thereby accomplishing rapid vascular effects. Penetration of arachidonate or newly-formed prostaglandins to cortical nerve-endings is inevitably made difficult by the abundance of cortical prostaglandin dehydrogenase. Evidence of a postjunctional site of action is indicated by De la Lande et al. (1975). They found that arachidonate depressed vasoconstrictor responses of the isolated rabbit ear artery to both NS and NA, and, that this depression was abolished in the presence of indomethacin (3 µg/ml). Ryan and Zimmerman (1973, 1974) report a depression by arachidonic acid (1-2 µg/ml) of vasoconstrictor responses to NA in the dog paw, again a postjunctional effect.

Contrary to the evidence discussed in favour of a postjunctional action, or a combination of pre- and postjunctional actions of arachidonate, Malik and McGiff (1975) provide challenging results in support of a primary prejunctional action. A concentration of 10 ng/ml arachidonic acid was sufficient to significantly reduce vascular responses to NS without altering equiconstrictor responses to NA, in their experiments on the isolated rabbit kidney. The capacity of

arachidonic acid to affect transmitter release in such low concentrations in these experiments, when 300 ng/ml had no effect in present experiments, is indeed inexplicable.

(iii) Influence of arachidonate on adrenergic transmitter overflow in response to RNS

Experiments in this section furnish conclusive evidence that arachidonate is capable of reducing the overflow of adrenergic transmitter resulting from RNS, without affecting resting transmitter overflow. Furthermore, this effect is almost completely suppressed by indomethacin, indicating that it results from de novo synthesis of prostaglandins, rather than an action of the arachidonate per se.

GENERAL DISCUSSION

In the first section of this thesis experiments are described which were designed in the hope of answering the first of the questions posed in the General Introduction: do prostaglandins which are released from the kidney on nerve stimulation originate presynaptically from adrenergic nerve endings or postsynaptically? It was proposed that if both NA and RNS could stimulate prostaglandin release, the site of prostaglandin release was most probably post-synaptic. Davis and Horton (1972) have demonstrated that RNS increases prostaglandin output in renal venous blood of rabbits. In Section 1, it was shown by two methods of prostaglandin analysis that noradrenaline also increases the release of prostaglandins into renal venous blood of rabbits. These results, in conjunction with additional evidence cited in the discussion of Section 1, are indicative of a post-junctional site of release of prostaglandins in the kidney, although a prejunctional site of release cannot be excluded at this stage. The recent finding that the α-adrenoceptor blocker, phenoxybenzamine, can selectively block either postjunctional or both prejunctional and postjunctional α-receptors, according to the dose-level employed (Dubocovich and Langer, 1974), will hopefully lead to more definitive studies on the site of release of prostaglandins.

The ability of the kidney to maintain constant blood flow in face of changing perfusion pressure i.e. to auto-regulate, is thought to be partly explicable in terms of a redistribution of cortical blood flow. Thus a reduced

outer cortical blood flow and an augmented flow to the inner cortex have been observed during haemorrhagic hypotension in dogs (Carriere, Thorburn, O'Morchoe and Barger, 1966; Truninger, Rosen, Grandchamp, Strebel and Kriek, 1971; Hardaker, Graham and Wechsler, 1975). Similarly, graded aortic constriction (McNay and Abe, 1970) and splanchnic nerve stimulation (Pomeranz, Birtch and Barger, 1968) have been shown to decrease outer cortical and increase inner cortical blood flow. Several pieces of evidence favour PGE as a possible mediator of renal autoregulation. Firstly, Itskovitz et al. (1974) observed that redistribution of renal blood flow to the inner cortex was associated with a rise in efflux of a PGE-like substance from the isolated perfused kidney. When indomethacin was added to the perfusate to block prostaglandin synthesis, a decrease in total renal blood flow and, in particular, inner cortical blood flow was observed.

Increased synthesis of endogenous prostaglandin in the kidney resulting from infusion of the major renal prostaglandin precursor, arachidonic acid, also affects renal blood flow distribution. Arachidonate infusion into the aorta of the rabbit (Larsson and Änggård, 1974) and into the renal artery of the dog (Chang et al., 1975) both increased perfusion of the inner relative to the outer renal cortex. Thus prostaglandins seem to influence intrarenal blood flow distribution.

The role of renal nerves and circulating catecholamines in the control of renal blood flow and its distribution is

unclear. Although both NA and RNS induce a redistribution of renal blood flow, they are apparently not essential to the redistribution occurring during haemorrhagic hypotension. Hardaker et al. (1975) have shown that redistribution under these circumstances occurs even in denervated kidneys or when dogs are reserpinised. In experiments on the isolated (and hence denervated) canine kidney, Itskovitz et al. (1974) also observed redistribution of renal blood flow. However, it has been reported that autoregulation is less efficient in the absence of renal nerves (see Wesson, 1969 for references). Since both the outer and inner cortices of the kidney appear to be innervated, a mechanism must exist whereby blood flow can be maintained through the inner cortex during periods of intense sympathetic stimulation. If, as is suggested, prostaglandins are responsible for maintenance of inner cortical blood flow, the questions arise as to whether they act by (1) producing a direct vasodilatation in this region (2) influencing vascular responsiveness to catecholamines (circulating or released by RNS) or (3) decreasing the effectiveness of RNS by reducing transmitter output. These questions encompass questions (ii) and (iii) posed in the General Introduction. The results obtained in Section 2 indicate that PGE_2 is the most likely mediator of blood flow maintenance in the kidney. The concentration of PGE_2 required to produce an effect on perfusion pressure and/or vascular responsiveness to adrenergic stimuli was similar to the highest concentration observed in renal venous blood following noradrenaline infusion

(Section 1) - about 50 ng/ml (ca. 3 x 10^{-7}M). The requirement of doses at least as high and higher than the maximum output concentration to produce effects when infused intra-arterially to the isolated kidney is probably the consequence of partial metabolism of PGE_2 in the renal cortex before it has an opportunity to reach its site of action. From the results obtained in Section 2, it was concluded that PGE_2 produced an effectively greater inhibition of vascular responses to RNS than of those to NA. Consequently, it was proposed that PGE_2 might produce this differential effect by acting prejunctionally as well as postjunctionally, at the adrenergic synapse. The results presented in Section 3 provide evidence that PGE_2 <u>does</u> act prejunctionally to reduce adrenergic transmitter release.

The regionalisation of prostaglandin synthesis (mainly in the medulla) and metabolism (mainly in the cortex) suggests a functional separation of the two systems. If prostaglandins of the E series are involved in the control of adrenergic transmission in the kidney, they must be able to gain access to the cortical nerve terminals. Such access is feasible in view of the findings that PGE_2 <u>is</u> synthesised in the cortex although to a lesser extent (one tenth) than in the medulla. In order to study effects of endogenous prostaglandin (and thus its ability to gain access to adrenergic terminals) on adrenergic transmission (question (iv), General Introduction), experiments were conducted in which endogenous prostaglandin synthesis was either decreased (with indomethacin) or increased (with arachidonate). The results obtained from these experiments

(Sections 4a and 4b) showed that a decrease in prostaglandin synthesis could enhance transmitter release whilst an increase in prostaglandin synthesis reduced transmitter release. These results are entirely compatible with the view that endogenous prostaglandins (mainly PGE_2) control adrenergic transmitter release in the kidney and also indicate that cortical nerve endings are accessible to endogenously-produced prostaglandin. It seems probable that the prostaglandin acting on adrenergic transmission is synthesised locally to (or perhaps within) the adrenergic nerve endings, in the renal vasculature itself. Prostaglandin synthesis by vascular smooth muscle has been demonstrated using various blood vessel types. Terragno, Crowshaw, Terragno and McGiff (1975) have demonstrated that bovine mesenteric arteries and veins share, with equal capacity, the ability to synthesise prostaglandins. Prostaglandins were assayed in these experiments by the superfusion technique. Separation by thin layer chromatography indicated that, under control conditions, synthesis of PGE_2 was twice that of $PGF_{2\alpha}$. Kalsner (1975) investigated the prostaglandin synthetic capacity of bovine coronary arteries and found that, on incubation in Krebs solution, they synthesised large amounts of a PGE-like substance. Inhibition of prostaglandin synthesis with aspirin, indomethacin or ETA led to a sustained contraction of the arteries suggesting that endogenous PGE production determines the degree of relaxation of these arteries. These observations on isolated preparations clearly provide evidence that prostaglandins can alter vascular smooth muscle tone in the absence

of sympathetic nervous activity, presumably by a direct myogenic action.

An interesting observation was made by Rioux and Regoli (1975) who were studying the release of prostaglandins from thoracic aortae of hypertensive and normal rats. These authors found that aortae from hypertensive rats released higher amounts of prostaglandin than those from normal rats. The results led them to the conclusion that the absence of relaxation of aortic strips from hypertensive rats might be due to increased intramural synthesis and release of prostaglandins. This is an interesting finding in view of the species differences previously described in Section 2, namely that PGE_2 causes vasoconstriction in the rat kidney but vasodilatation in the rabbit kidney. Thus in the rat, PGE may promote hypertension.

Possible mechanisms by which prostaglandins might alter adrenergic transmitter release

It is now well-known that α-adrenoceptor blocking drugs increase the release of adrenergic transmitter as shown in early experiments by Brown and Gillespie (1957). Considerable evidence has since accumulated showing that NA release is controlled by a negative feedback of noradrenaline itself on adrenergic nerve endings to prevent or reduce further transmitter release. Thus, by blocking the α-receptors which exert this control (thought to be prejunctionally located), the braking effect is lost and trans-

mitter release increases (for references, see Usdin and Snyder, 1973; Burnstock and Costa, 1975). Bearing in mind the α-mediated control of noradrenaline release, some workers have explored the role of endogenous prostaglandins in the control of transmitter release and have attempted to find out whether they act within the α-mediated system or exert an independent control.

Hedqvist demonstrated that the α-blocking drugs phentolamine and phenoxybenzamine increase adrenergic transmitter release (gauged using ^{3}H-NA) from the transmurally-stimulated guinea pig vas deferens. Following inhibition of prostaglandin synthesis with ETA, these compounds still caused an increase in transmitter overflow. This effect was abolished by PGE_2. Noradrenaline also inhibited ^{3}H-NA overflow in the presence of ETA. Thus it may be concluded that the α-mediated control system can be triggered in the absence of endogenous prostaglandin, suggesting that the two control mechanisms operate independently of each other (Hedqvist, 1974c). In another set of experiments, Hedqvist found, conversely, that α-blockade did not significantly affect the inhibitory action of prostaglandin (Hedqvist 1974a).

Stjärne has further demonstrated that the effect of noradrenaline on ^{3}H-NA overflow (to decrease it) from the field-stimulated guinea pig vas deferens was not significantly altered by incubation of the tissue with ETA, although the enhancing effect of ETA on ^{3}H-NA overflow was dose-dependently depressed by the α-blocker, phentolamine. These

results suggest that the two control mechanisms, α-mediated and PG-mediated, might act independently but that the release of endogenous prostaglandin is itself α-mediated. Thus α-receptors trigger two feedback control mechanisms, one operated by NA and one by PGE (Stjärne, 1973b, 1973d). Two independent mechanisms of control of adrenergic transmitter release, one PG-mediated and one α-mediated, have also been proposed to exist in the rabbit heart (Starke and Montel, 1973), although in these experiments there was no indication that the PG-mechanism operated via stimulation of α-receptors. A role for prostaglandins in the control of NA release has also recently been extended to humans. Stjärne and Gripe (1973) have shown that the dual α/PG control mechanisms which appear to control transmitter release in the vas deferens, also operate in human peripheral arteries and veins. Studies were carried out on isolated segments of these vessels, obtained by biopsy from normotensive individuals.

The release of adrenergic transmitter is critically calcium-dependent (see Hubbard, 1970). It is interesting, therefore, that a relationship between calcium and the inhibitory effect of PGE_2 in adrenergic neurotransmission has been established. Hedqvist (1970c) demonstrated that when the calcium concentration of the medium used to perfuse the isolated cat spleen was raised from 2.5 mM to 5.0 mM, the outflow of NA in response to splenic nerve stimulation (NS) was increased. Furthermore the increased calcium concentration strongly counteracted the inhibitory effect of

PGE_2. A similar reversal of the inhibitory action of PGE_2 was observed by raising the external calcium concentration from 1.8 mM to 5.4 mM in the superfusion fluid of the isolated field-stimulated guinea pig vas deferens (Hedqvist, 1974b). PGE_2 was still inhibitory in these experiments but its effect was less marked, with a shift to the right of the dose-inhibition curve. Stjärne also demonstrated, using the vas deferens preparation, that the inhibitory effect of PGE_2 varies inversely with the external calcium concentration. He suggested that PGE might facilitate efflux of intraaxonal calcium (Stjärne, 1973a) since other workers have shown that PGE may act as a calcium ionophore, facilitating calcium movement across biological membranes (Kirtland and Baum, 1972). An alternative suggestion made by Stjärne, in a kinetic study of the calcium dependence of the NA secretory process, was that PGE_2 might depress the affinity for calcium of the secretory mechanism (Stjärne, 1973c). It must be concluded, however, that the exact mechanism whereby PGE inhibits adrenergic transmitter release is not, as yet, fully understood.

Despite the present lack of understanding of the mechanism of action of PGE, its effect on renal adrenergic neurotransmission has considerable implications in the field of renal hypertensive disorders. If the normotensive state is dependent upon the continued production of PGE_2 to control renal vascular tone, it is conceivable that some hypertensive states might result from a lack of, or reduction, in renal prostaglandin production. PGE_2,

itself, would be rather unsuitable as a drug for treatment of hypertension for reasons of its rapid metabolism by the lungs. Various prostaglandin analogues have been synthesised recently, however, which show greater biological activity and greater stability in the circulation than PGE_2 e.g. 16,16'-dimethyl-PGE_2. The development of such substances will hopefully lead to their eventual application in the treatment of renal vascular disorders.

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EFFECT OF PROSTAGLANDIN E_2 ON VASCULAR RESPONSES OF THE RABBIT KIDNEY TO NERVE STIMULATION AND NORADRENALINE, IN VITRO AND IN SITU

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Summary

The effect of prostaglandin E_2 on vascular responses of the rabbit kidney to renal nerve stimulation and noradrenaline was examined in vitro and in situ as a test of the hypthesis that prostaglandins of the E series may be involved in the regulation of adrenergic neuroeffector transmission. Intraarterial administration of prostaglandin E_2 to the in vitro kidney caused marked inhibition of vascular responses to nerve stimulation whereas the responses to noradrenaline were not significantly altered. In the in situ preparation, vascular responses to both nerve stimulation and noradrenaline were inhibited by prostaglandin E_2 infusion, although its effect on responses to nerve stimulation was approximately twice that observed on responses to noradrenaline.

It is concluded that prostaglandin E_2 acts primarily at a prejunctional level of adrenergic neuroeffector transmission in the kidney, although a postjunctional effect has also been observed.

Introduction

The purpose of the present study was to examine the hypothesis that prostaglandins of the E series may be involved in the regulation of transmitter release from sympathetic nerve endings (4). The isolated rabbit kidney with renal nerve dissected free was used as a model in most experiments although some in situ experiments were included in the study as a check on the validity of the in vitro situation. It has already been shown that large amounts of prostaglandin E_2 (PGE_2) are present in renal venous blood of rabbits, and that this output is increased on renal nerve stimulation (2). It seems likely, therefore, that this increased output of PGE_2 during nerve

stimulation may serve some regulatory function by affecting further transmitter release from renal sympathetic nerve endings.

The present experiments consisted of administration of PGE_2 to the kidney intraarterially, and observation of its effects on renal vascular responses to nerve stimulation (NS), and exogenous noradrenaline (NA).

Methods

a) In vitro experiments

20 rabbits weighing 2-2.5 kg were anaesthetized with 25 % urethane, 7 ml/kg. The abdomen was opened along its midline and the left kidney, with its nervous and vascular supply, was dissected free from the surrounding tissue. The nerve was then carefully freed from the artery. After heparinisation (1I.U./g. body weight) the vessels and ureter were cannulated, the nerve was cut and the kidney was flushed with warm 0.9 % saline containing 50 I.U. heparin/ml. The preparation was then transferred immediately to a perfusion chamber maintained at 37°C and was perfused with Tyrode solution (concentrations in mM: NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5) also at 37°C, containing 2 % dextran and gassed with 5 % CO_2 in O_2.

Perfusion flow was kept constant at a rate of 10 ml/min and pressure was recorded close-arterially to the kidney on a Grass Model 5 Polygraph using a Statham (P 23 AC) pressure transducer.

The nerve was placed on platinum electrodes and stimulated with a Grass S5 stimulator delivering 15-30 sec trains of pulses (6-10 Hz, 2 msec, 5-15 V) at 10 min intervals.

NA and PGE_2 were infused intraarterially in Tyrode solution.

b) In situ experiments

4 rabbits were anaesthetized as before and the left renal nerve was freed from the artery. Heparinisation of the animal was maintained by administration of 1 I.U./g. body weight every two hours. The left kidney was perfused with blood looped from the carotid artery through a constant rate pump, adjusted so that the renal arterial pressure approximated normal systemic pressure (90-110 mm Hg). This procedure required flow rates ranging from 5-7 ml blood/min and facilitated the administration of drugs at constant concentration, as in the in vitro studies.

The renal vascular pressure was measured as before and drugs were administered in Tyrode solution of minimal volume.

Results

a) In vitro experiments

It was consistently observed that doses of PGE_2 ranging from 3.8×10^{-7} M to 1.5×10^{-6}M, which did not alter the basal renal arterial pressure on infusion, caused significant inhibition of vascular responses to nerve stimulation (NS, Fig 1). There was partial or complete recovery from this inhibition 10 - 20 min after ending the infusion of PGE_2. At dose-levels below 1.5×10^{-7}M, PGE_2 showed no significant effect on responses to NS in most cases, although weak inhibition or even slight enhancement of responses was occasionally observed.

At higher dose-levels, (3.8×10^{-7} - 1.5×10^{-6}M) the inhibitory effect of PGE_2 was found to be statistically significant at the 2 - 0.1 % level, according to Student's t-test for paired variates (Fig 1).

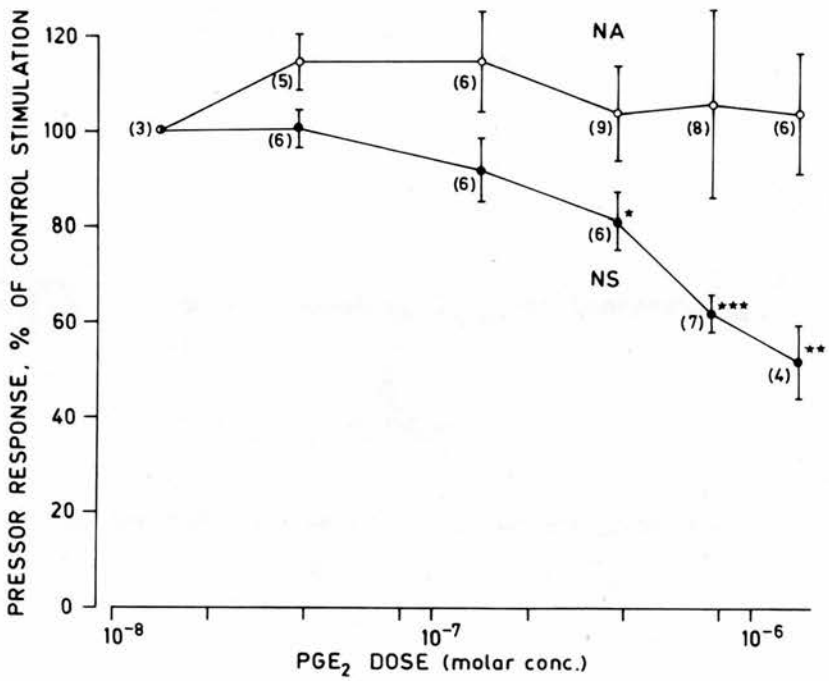

FIG 1

Isolated, perfused rabbit kidneys. Effect of different doses of PGE_2 on pressor responses to renal nerve stimulation, NS (200 pulses, 10 Hz, 2 msec, 7-10 V) and to noradrenaline, NA (0.26 - 0.52 µg/ml, 20 sec infusions). All values presented as per cent of a preceding control stimulation. Vertical bars = mean values ± SEM. Figures within brackets = no of expts. * $p<.05$, ** $p<.01$, *** $p<.001$ according to Student's t-test for paired variates.

In contrast to its effect on responses to NS, PGE_2 in the same concentrations did not produce well-defined, dose-dependent effects on vascular responses to NA. The most commonly observed effect was that of enhancement of responses although in some experiments either no effect or moderate inhibition was observed.

Although the mean values (responses as % control level) were all at or above control level, a statistical analysis showed that the mean responses to NA were not significantly altered from the control level by PGE_2 (Fig 1).

b) In situ experiments

As observed in vitro, PGE_2 consistently inhibited vascular responses of the kidney to NS, with complete recovery from inhibition occurring about 10 min after ending the PGE_2 infusion. The in situ kidney proved more sensitive to the inhibitory effect of PGE_2 than that in vitro, marked inhibition of NS responses being produced even in the lower dose-range, 1.5×10^{-7}M - 3×10^{-7}M (Fig 2).

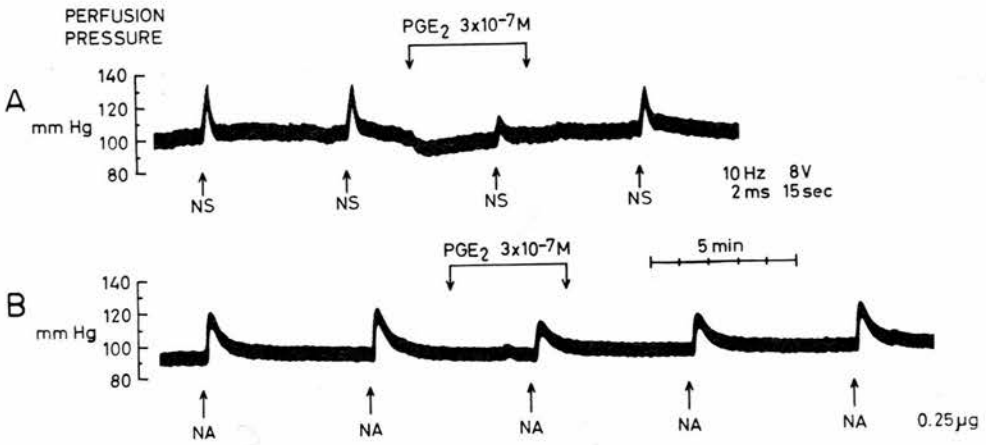

FIG 2

Blood perfused rabbit kidney in situ. Effect of PGE_2 on pressor responses to renal nerve stimulation (A) and to noradrenaline (B) in one and the same experiment.

Within the same dose-range, vascular responses to NA were also inhibited by PGE_2, although the inhibition was much less marked than that observed on responses to NS.

Discussion

The present study has shown that PGE_2 inhibits responses of the rabbit kidney to NS both *in vitro* and *in situ*. The quantitative difference in effectiveness of PGE_2 in the two preparations may be attributable to the fact that a higher stimulation frequency (10 Hz) was required to produce suitable pressor responses in the *in vitro* preparation than was required *in situ* (5 - 8 Hz). It has been demonstrated that the inhibitory effect of PGE_2 on responses (of the vas deferens) to field stimulation diminishes as the frequency of stimulation is increased (5). The present results are in agreement with those reported by Lonigro *et al.* (8) on the *in vivo* dog kidney.

The discrepancy between effects of PGE_2 on renal vascular responses to NA *in vitro* and *in situ* is not entirely surprising in view of the many conflicting results which have been reported by other workers. Davies and Withrington (1) found no effect of PGE_2 on vascular responses of the isolated dog spleen to NA whilst in the isolated cat spleen, the vascular responses to NA were found to be inhibited by low doses of PGE_2 6×10^{-8} - 3×10^{-7} M and potentiated by high doses, 6×10^{-6} M (3).

The *in situ* dog kidney was studied by Kadowitz *et al.* (6), who found that PGE_1 caused inhibition of vascular responses to NA. It is difficult to extrapolate from such results since PGE_1 was used instead of PGE_2, although the two prostaglandins commonly show qualitatively similar effects. The present *in situ* studies are, however, in complete agreement with those of Lonigro *et al.* (8), who have shown that PGE_2 inhibits responses of the *in vivo* dog kidney to NA but that this inhibition is weaker than that produced on similar responses evoked by NS.

McGiff and Itskovitz (10) suggest that use of dose-levels of PGE_2 exceeding 100 ng/ml blood is questionable. In view of the location of sympathetic nerve endings being presumably confined to the cortex, it seems justifiable to have used even higher doses of PGE_2, since PGE_2 is rapidly metabolized by prostaglandin dehydrogenase in the rabbit renal cortex (7). Furthermore, the output of PGE_2/ml renal venous blood is considerably higher in the rabbit than in the dog (2, 9).

Results of the present studies *in vitro* would seem to suggest that PGE_2 is producing its inhibitory effect on vascular responses to NS mainly at the pre-junctional level, since its post-junctional effects on NA responses are

apparently negligible. The in situ studies, however, point to some post-junctional inhibitory action of PGE_2. It is unlikely that such an action can account for the total inhibitory action of PGE_2 on NS, since inhibition of NA responses approximates only 50 % of that observed at the same PGE_2 dose-level on NS.

In conclusion, it is suggested that PGE_2 has some pre-junctional effect on the sympathetic nerve endings in the present system. Further studies on transmitter release from renal nerve endings are required to confirm this suggestion.

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ffects of Prostaglandin E_2 on the Sympathetic Neuroeffector System of the Rabbit idney in Vitro and in Situ

By MADELINE H. FRAME and PER HEDQVIST. *From the Department of Physiology, Karolinska Institute, Stockholm, Sweden*

t has already been shown that large amounts of prostaglandin E_2 (PGE_2) are resent in the renal venous blood of rabbits and that this output is increased on renal erve stimulation (Davis and Horton 1972). The present study was carried out to xamine the hypothesis that PGs of the E series may be involved in the regulation f transmitter release from sympathetic nerve endings (Hedqvist 1973). The isolated abbit kidny with renal nerve dissected free was used as a model in most experiients although some *in situ* experiments were included in the study as a check on he validity of the *in vitro* situation.

Experiments were carried out to observe the effect of intra-arterially infused PGE_2 on (a) vascular response of the kidney to nerve stimulation and exogenous oradrenaline, and (b) overflow of tritium-labelled noradrenaline during nerve timulation.

In the (a) experiments, infusion of PGE_2 (63—520 ng/ml) into the *in vitro* idney caused marked inhibition of vascular responses to nerve stimulation, whereas he responses to noradrenaline were not significantly altered. In the *in situ* preparaion, however, vascular responses to both nerve stimulation and noradrenaline were nhibited by PGE_2 infusion, although its effect on responses to noradrenaline was pproximately half of that observed on responses to nerve stimulation.

In the (b) experiments, infusion of PGE_2 (63—320 ng/ml) into the *in vitro* idney caused consistent reduction in overflow of labelled transmitter during nerve timulation. In addition, it was found that this inhibitory effect of PGE_2 varied inersely with the stimulation frequency, total number of pulses being kept constant n every stimulation.

In both series of experiments, the inhibitory effect of PGE_2 was dose-dependent nd partial or complete recovery from this effect occurred within 10 to 20 min. he necessity for use of relatively high doses of PGE_2 seemed permissible in view of ne rapid metabolism of PGs by prostaglandin dehydrogenase in the rabbit renal ortex (Larsson and Änggård 1973).

It is concluded that PGE_2 acts at both prejunctional and postjunctional levels of drenergic neurotransmission in the rabbit kidney.

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EVIDENCE FOR PROSTAGLANDIN MEDIATED PREJUNCTIONAL CONTROL OF RENAL SYMPATHETIC TRANSMITTER RELEASE AND VASCULAR TONE

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1 Prostaglandin E_2 dose-dependently and reversibly inhibited the noradrenaline overflow resulting from nerve stimulation of the rabbit kidney.

2 The magnitude of this inhibition varied inversely with the frequency of stimulation employed.

3 The prostaglandin synthesis inhibitors, indomethacin and meclofenamic acid, both increased the transmitter overflow resulting from renal nerve stimulation, suggesting that endogenous prostaglandin has a role in the regulation of transmitter release.

4 In the presence of indomethacin, the inhibitory effect of exogenous prostaglandin E_2 was enhanced.

5 The prostaglandin precursor, arachidonic acid, also caused a significant, dose-dependent and reversible inhibition of transmitter overflow. This inhibition became insignificant when arachidonic acid was applied in the presence of indomethacin, suggesting that the inhibition was mediated by newly formed prostaglandin rather than by arachidonic acid itself.

6 It is proposed that newly formed prostaglandin controls noradrenaline release primarily from inner cortical nerve endings, thereby maintaining juxtamedullary blood flow under periods of increased sympathetic nerve activity.

Introduction

The rabbit kidney has a high capacity for both synthesis (predominantly in the medulla) and degradation (in the renal cortex) of prostaglandins (Hamberg, 1969; Crowshaw, 1971; Larsson & Änggård, 1973). Prostaglandin E_2 is the most abundant renal prostaglandin and is a potent vasodilator (Lee, Crowshaw, Takman, Attrep & Gougoutas, 1967; Daniels, Hinman, Leach & Muirhead, 1967). Consequently, it has been postulated that this prostaglandin has a physiological role in blood flow regulation in the kidney (McGiff, Crowshaw, Terragno & Lonigro, 1970; Lonigro, Terragno, Malik & McGiff, 1973; McGiff & Itskovitz, 1973; Herbaczynska-Cedro & Vane, 1973, 1974; Larsson & Änggård, 1974; Needleman, Douglas, Jalsetik, Stoecklein & Johnson, 1974). It has in addition been shown that both renal nerve stimulation (RNS) and catecholamine administration increase the output of prostaglandins in the renal venous effluent of rabbits, prostaglandin E_2 being most abundant (Davis & Horton, 1972; Needleman *et al.*, 1974).

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In the light of previous evidence which suggests a role for prostaglandin E_2 in regulation of transmitter release from sympathetic nerve endings (Hedqvist, 1970, 1973, 1974a, b), it was decided to examine the possibility of such an interaction in the rabbit kidney. Previous experiments have shown that prostaglandin E_2 has an inhibitory effect on vascular responses of the rabbit kidney to RNS *in vitro* and *in situ* and that this inhibition is probably, in part, prejunctional (Frame, Hedqvist & Åström, 1974; Frame & Hedqvist, 1974).

The aim of the present study was to assess the capacity of prostaglandin E_2, prostaglandin precursor (arachidonic acid) and prostaglandin synthesis inhibitors (indomethacin, meclofenamic acid) to affect transmitter overflow resulting from RNS.

Methods

Forty rabbits weighing 2 to 3 kg were anaesthetized with 25% urethane, 7 ml/kg. The abdomen was opened along its midline and the left

kidney, with its nervous and vascular supply, was dissected free from the surrounding tissue. The nerve was then carefully freed from the artery. After heparinization (1000 i.u./kg, i.v.) the vessels and ureter were cannulated, the nerve was cut and the kidney was flushed with 0.9% w/v NaCl solution (saline) containing 50 i.u. of heparin per ml. The preparation was then transferred to a perfusion chamber maintained at 37°C and was perfused with Tyrode solution (mM: NaCl 136.7, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, ascorbic acid 0.1) also at 37°C, containing 2% dextran and gassed with 5% CO_2 in O_2.

The renal perfusion rate was kept constant at 10 ml/min and pressure was recorded on a Grass Model 5 polygraph using a Statham P 23 AC pressure transducer.

Noradrenaline (NA) stores were labelled by infusion of 25-50 μCi of tritiated NA in Tyrode solution over a period of about 20 minutes. The kidney was then perfused with isotope-free Tyrode solution for 30 min to remove surplus tritiated NA.

The nerve was placed on platinum electrodes and stimulated with a Grass S5 stimulator delivering 15 to 75 s trains of pulses (2-10 Hz, 2 ms, supramaximal voltage) at 10 min intervals. The total number of pulses applied per stimulation period was kept constant regardless of frequency. The perfusate was collected in 1 min fractions for 2 min prior to nerve stimulation, during stimulation and for 2 min post-stimulation. The tritium content of the perfusate was determined by counting 0.5 ml aliquots in a Packard liquid scintillation spectrometer with quenching monitored by internal standards. Twenty ml of a 3 : 7 ethanol-toluene mixture containing 4 g of 2-5 diphenyloxazole (PPO) and 0.1 g of 1-4 di (2-(4-methyl-5-phenyloxazolyl))benzene (POPOP) per litre of toluene was used as a counting medium for each sample.

The radioactivity of the perfusate samples was separated into that of intact NA and that of its metabolites by cation exchange column chromatography (Fredholm & Hedqvist, 1973). The recovery of authentic NA added to the samples and carried through the entirely chromatographic procedure was 73.3 ± 2.3% (mean ± s.e. mean, $n = 4$).

The following drugs were used: arachidonic acid, Sigma Chemicals, St. Louis, U.S.A.; [^{3}H]-(−)-noradrenaline, 7.2 Ci/mmol, The Radiochemical Centre, Amersham; indomethacin was a gift from Merck, Sharpe & Dohme, Rahway, N.J., U.S.A.; meclofenamic acid from Parke Davies, Pontypool, Wales, and prostaglandin E_2 from Dr J. Pike, Upjohn Co., Kalamazoo, Mich., U.S.A.

Arachidonic acid (99%, porcine liver) was further purified by silicic acid column chromatography shortly before use. All drugs were administered close-arterially to the kidney in Tyrode or saline solution.

Results

Stimulation of the renal nerves (2-10 Hz, 2 ms duration, supramaximal voltage) caused a marked increase in overflow of radioactivity from the kidney, which was accompanied by an increase in the perfusion pressure. During RNS, intact [^{3}H]-NA accounted for 81.3% of the radioactivity, compared to 14.4% during resting periods. In contrast, material not adsorbed to the column (mainly deaminated products) accounted for only 16.8% during RNS but 74.4% during resting periods. The content of [^{3}H]-normetanephrine, which made up 10% of the radioactivity during rest decreased to less than 2% during stimulation (Table 1).

Effect of exogenous prostaglandin E_2

It was consistently found that infusions of prostaglandin E_2 ($1.8\text{-}9.1 \times 10^{-7}$M) 2 min prior to and during RNS, which did not alter the basal renal arterial pressure, decreased the overflow of transmitter in response to RNS in a dose-dependent manner (Figure 1). The degree of inhibition of [^{3}H]-NA overflow varied inversely

Table 1 Cation exchange column chromatography of radioactivity appearing in venous effluent of rabbit kidney previously loaded with [^{3}H]-noradrenaline.

Sample	*No. of expts.*	*'Acid metabolites'*	*Noradrenaline*	*Normetanephrine*
Stimulated	7	16.8 ± 2.3	81.3 ± 2.2	1.3 ± 0.4
Resting	5	74.4 ± 2.3	14.4 ± 1.1	10.0 ± 1.5

Perfusate samples withdrawn before and during nerve stimulation. Chromatographic values presented as relative distribution (per cent) of recovered 'acid metabolites' (mainly deaminated products), intact noradrenaline and normetanephrine, and given as means ± s.e. mean.

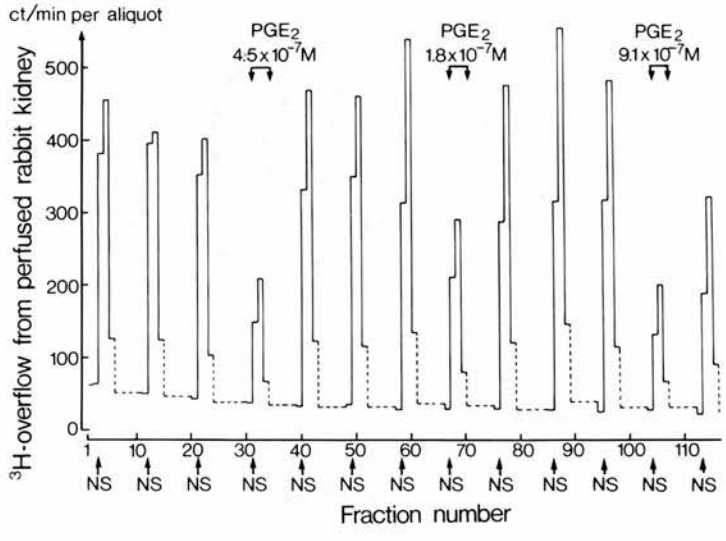

Figure 1 Isolated, perfused kidney of the rabbit, preloaded with [^{3}H]-noradrenaline. Effect of prostaglandin E_2 (PGE_2) on overflow of tracer resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz. Time in min = fraction numbers. Dotted lines represent uncollected fractions.

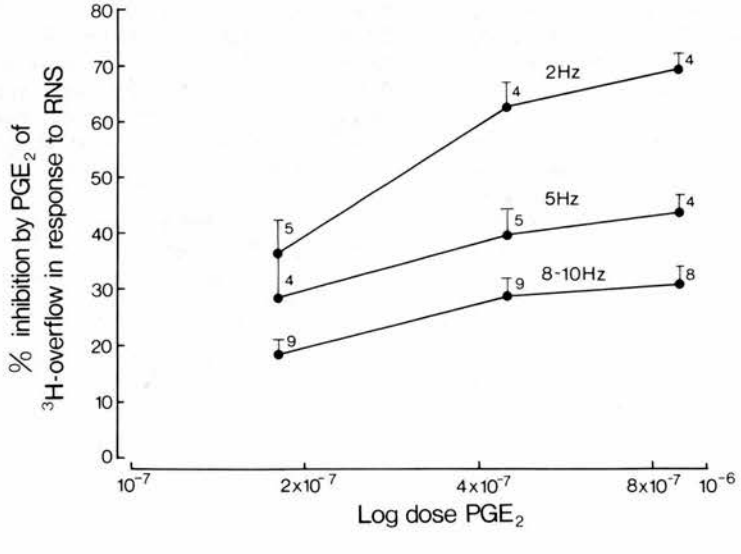

Figure 2 Inhibitory effect of prostaglandin E_2 (PGE_2) on transmitter overflow resulting from renal nerve stimulation (RNS), 150 pulses, 2-10 Hz. Vertical bars show s.e. mean. Figures at the points = number of observations.

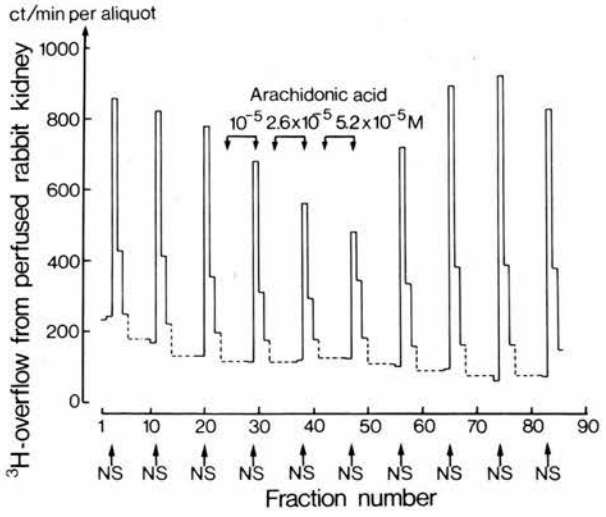

Figure 3 Effect of cumulative doses of arachidonic acid on transmitter overflow resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz.

with the stimulation frequency employed (Figure 2). The vascular responses to RNS were similarly inhibited and there was partial or complete recovery from both [^{3}H]-NA overflow and vascular-inhibition 10 min after ending the infusion of prostaglandin E_2.

Effect of arachidonic acid

When arachidonic acid (2.6×10^{-5}M) was infused 5 min prior to and during RNS, transmitter overflow was inhibited by 35.3% (see Table 2). On the addition of cumulative doses of arachidonic acid, a dose-dependent inhibition was observed (Figure 3). Concomitantly there was a corresponding reduction of the vascular response to RNS. In most of the experiments, a slight reduction in perfusion pressure was observed throughout the duration of the infusion. Partial or complete recovery from the inhibition usually occurred within 10-20 min of ending the arachidonic acid infusion, although in some experiments the transmitter overflow remained depressed.

Effect of prostaglandin synthesis inhibitors

Indomethacin was used to block the synthesis of endogenous prostaglandins (Vane, 1971) and thus to evaluate the possible role of the latter in adrenergic transmission in the kidney.

At a stimulation frequency of 5 Hz (150 pulses), infusion of indomethacin (4.4×10^{-5}M) sometimes caused a slight increase in the resting perfusion pressure (up to 5 mmHg) and either no effect or a slight decrease in the effector responses to RNS. The effect on transmitter overflow, however, was much more marked. The overflow of [^{3}H]-NA during RNS increased significantly 8 min after the start of an infusion of indomethacin and plateaued after about 15 min of infusion. The maximal increase of [^{3}H]-NA overflow in response to RNS was $41.5 \pm 7.0\%$ (mean ± s.e. mean, $n = 13$). This value was significant at the $P < 0.001$ level according to Student's t-test for paired varieties. After ending the infusion, the transmitter overflow fell off approaching the preinfusion level within 10 to 20 min. However, in some experiments the transmitter overflow remained at an elevated level throughout post-infusion controls (Figure 4).

In three additional experiments, meclofenamic acid, (5×10^{-5}M) which is also a potent inhibitor of prostaglandin synthesis (Gryglewski & Vane, 1972), was used instead of indomethacin. The increase in [^{3}H]-NA overflow in response to RNS produced by infusion of meclofenamic acid was $23.3 \pm 3.5\%$ ($P < 0.05$). The drug caused qualitatively the same effects as indomethacin, although it was less potent on an equimolar basis.

Effect of prostaglandin E_2 and arachidonic acid application during indomethacin treatment

During indomethacin treatment, prostaglandin E_2 was still effective in producing an inhibition of [^{3}H]-NA overflow in response to RNS. The

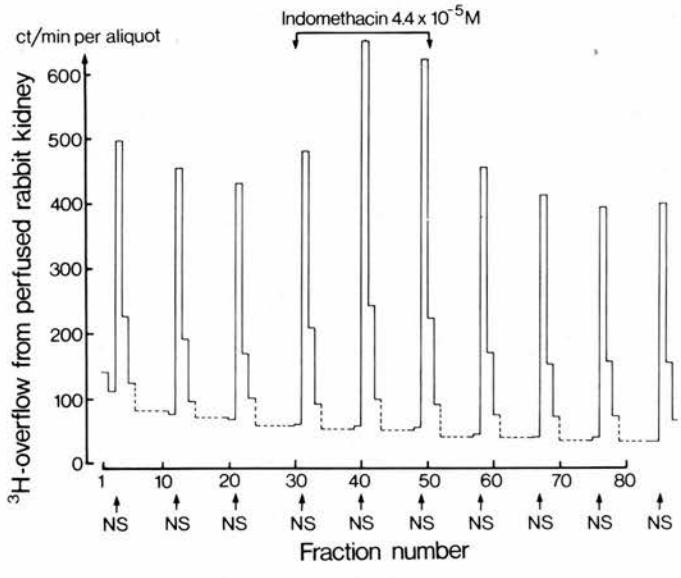

Figure 4 Effect of indomethacin on transmitter overflow resulting from renal nerve stimulation (NS), 150 pulses at 5 Hz.

inhibition produced was, in fact, significantly greater ($P < 0.02$) than that produced by prostaglandin E_2 alone, the values being 61.3% for indomethacin plus prostaglandin E_2 and 39.6% for prostaglandin E_2 alone (Table 2).

The inhibitory effect of arachidonic acid, unlike that of prostaglandin E_2, was significantly reduced ($P < 0.001$) from 35.3% for arachidonic acid alone to 10.6% for arachidonic acid in the presence of indomethacin. This would suggest that the inhibitory effect was primarily that of newly formed prostaglandin rather than that of the arachidonic acid itself (Figure 5, Table 2).

Discussion

It was previously shown that prostaglandin E_2 inhibits renal vascular responses to RNS and, to a lesser extent, exogenous NA in the dog and rabbit kidney (Lonigro *et al.*, 1973; Frame *et al.*, 1974). Those results merited the suggestion of a primary prejunctional action of prostaglandin E_2, as observed in other tissues (cf. Hedqvist, 1973).

The overflow of NA from an adrenergically innervated organ can be monitored by determining the efflux of [^{3}H]-NA previously taken up by the nerves (Hertting & Axelrod, 1961). Since in the

Table 2 Percentage change of transmitter overflow to RNS (5 Hz, 150 pulses) by prostaglandin E_2 (PGE_2) (4.5×10^{-7}M), arachidonic acid (2.6×10^{-5}M) and indomethacin (4.4×10^{-5}M), and by prostaglandin E_2 and arachidonic acid in the presence of indomethacin.

Administered compound	*Pretreatment*		*Difference*
	None	*Indomethacin*	*None − indomethacin*
PGE_2	−39.6 ± 4.5(5)	−61.3 ± 4.6(3)	21.7 ± 6.4
	$P < 0.001$	$P < 0.01$	$P < 0.02$
Arachidonic acid	−35.3 ± 4.0(5)	−10.6 ± 4.4(5)	24.7 ± 5.9
	$P < 0.001$	$P < 0.1$(NS)	$P < 0.001$
Indomethacin	+41.5 ± 7.0(13)	–	–
	$P < 0.001$		

Mean values ± s.e. mean, figures within brackets = number of experiments. Statistical analysis according to Student's *t*-test.

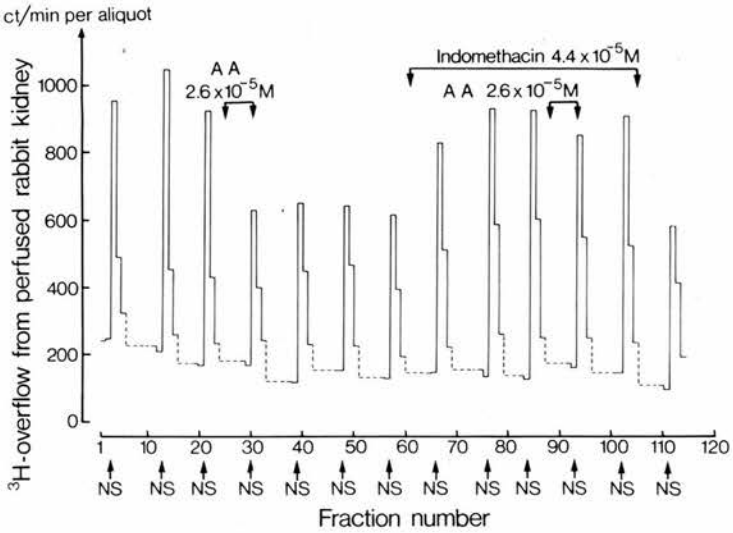

Figure 5 Effect of arachidonic acid (AA) alone and of arachidonic acid in the presence of indomethacin on transmitter overflow resulting from renal nerve stimulation (NS, 150 pulses at 5 Hz).

present study the majority of the radioactivity overflowing in response to RNS was represented by intact [^{3}H]-NA, total radioactivity was considered an appropriate indicator of endogenous NA overflow.

It is apparent from this study that transmitter overflow resulting from RNS is dose-dependently and reversibly inhibited by exogenous prostaglandin E_2 and can also be modified by altering endogenous prostaglandin levels. Judging from experiments on the cat spleen and the guinea-pig vas deferens and heart (Hedqvist, 1970, 1974a; Bhagat, Dhalla, Ginn, LaMontagne & Montier, 1972), where prostaglandin E_2 does not alter the disposition of released NA, it is likely that the observed inhibition of transmitter overflow represents an action directly on the process of excitation-secretion coupling in the nerve terminals.

In examining the inhibitory or 'braking' effect of exogenous prostaglandin E_2 on transmitter release, the control transmitter overflow to RNS acted as a guide to the effect of prostaglandin E_2. However, it could not be regarded as a true control since, at least in the dog and rabbit kidney, RNS causes release of prostaglandin E_2 which should also provide some braking action (Dunham & Zimmermann, 1970; Davis & Horton, 1972; McGiff, Crowshaw, Terragno, Malik & Lonigro, 1972). In an attempt to reduce the interference of endogenous prostaglandins, experiments were carried out in which prostaglandin synthesis inhibitors were administered to the kidney. The primary effect of the synthesis inhibitors, indomethacin and meclofenamic acid (Vane, 1971; 1973), was to elevate transmitter overflow, which is to be expected if an endogenous braking-mechanism has been partially or wholly removed. Moreover, in harmony with previous observations on the guinea-pig vas deferens (Hedqvist, 1974a), a given dose of prostaglandin E_2 showed an increased ability to inhibit transmitter overflow during indomethacin infusion. This increased potency may also be attributable to removal of a local braking mechanism, assuming that administered prostaglandin E_2 normally has to compete with endogenous prostaglandin for active sites in the nerve terminals.

In a series of experiments the inhibitory effect of prostaglandin E_2 on transmitter release was studied at different stimulation frequencies. Generally the inhibition produced by a given dose varied inversely with the stimulation frequency employed. This would seemingly suggest an endogenous prostaglandin-mediated braking mechanism which operates more readily at a low impulse activity than at a high. This is in good agreement with results obtained in the rabbit heart (Junstad & Wennmalm, 1973) where the sympathetic transmitter-release-mechanism became less sensitive to exogenous prostaglandin E_2 as the frequency of stimulation was increased from 2-10 Hz. The release of *endogenous* prostaglandin during sympathetic nerve stimulation of the dog

kidney and the rabbit heart shows no apparent linear correlation with either the frequency or the transmitter overflow (Dunham & Zimmerman, 1970; Junstad & Wennmalm, 1973). Thus an increase in stimulation frequency from 2-10 Hz only approximately doubled the efflux of prostaglandin E_2-like material per unit time whilst the transmitter overflow increased tenfold. It would seem, therefore, that the endogenous transmitter braking-mechanism is capable of compensating for changes in impulse activity but that its effectiveness is limited both by the amount of prostaglandin releasable and by the sensitivity of the NA-release process to prostaglandin.

The series of experiments in which the prostaglandin precursor arachidonic acid was used, was carried out in an attempt to show that newly synthesized prostaglandins have access to sympathetic junctions. Infusion of arachidonic acid produced a marked inhibitory effect on both transmitter overflow and vascular responses to RNS. This effect was mainly due to the formation of prostaglandins since the effect was very much reduced or abolished when arachidonic acid was infused in the presence of indomethacin. A small residual effect of the arachidonic acid is not unexpected, since indomethacin in doses similar to that used in the present study has been shown to inhibit markedly but not abolish the conversion of arachidonic acid to prostaglandin E_2 in rabbit kidney and in rat kidney homogenates (Larsson & Änggård, 1974; Fredholm, Hedqvist & Larsson, unpublished observations).

In discussing a role for prostaglandins as regulators of transmitter release at renal sympathetic nerve endings, the assumption is made that there is a ready source of prostaglandins in the vicinity of the cortical nerve endings. The biosynthesis of prostaglandins, however, has been shown to be ten times greater in the medulla than in the cortex (Larsson & Änggård, 1973). It has, nevertheless, been emphasized by these authors that cortical synthesis is large enough to be of significance. The renal cortex, in addition to its biosynthetic capacity, possesses an abundant amount of prostaglandin degrading enzyme, prostaglandin dehydrogenase (Änggård, Larsson & Samuelsson, 1971). It is probable, however, that endogenous cortical prostaglandin which escapes degradation may have access to the cortical nerve endings. This possibility is inhanced by the fact that prostaglandin E_2 infused into the present system, although going directly to the cortex, produced an effect on RNS. An alternative suggestion is that medullary prostaglandins may reach the juxtamedullary arterioles via the vasa recta (Larsson & Änggård, 1973).

Although both the juxtamedullary and outer cortical arterioles have adrenergic innervation, it is interesting that during RNS or induction of stress in rabbits, the outer cortex becomes ischaemic whilst juxtamedullary cortical blood flow persists (Trueta, Barclay, Daniel, Franklin & Pritchard, 1947). Moreover, the prostaglandin synthesis capacity of the cortex increases towards the medulla and stimulation of prostaglandin synthesis using arachidonic acid increases juxtamedullary blood flow in the rabbit (Larsson & Änggård, 1973; 1974). It seems, therefore, that prostaglandins are primarily available in the juxtamedullary zone to inhibit sympathetic arteriolar tone. It is apparent from the present study that this dilatation or prevention of vasoconstriction is, at least in part, attributable to inhibition of transmitter release, i.e. a prejunctional action on adrenergic neuro-effector transmission. However, part of the effect may be post-junctionally located at the effector cell level since prostaglandin E_2 to some extent inhibits vascular responses to NA in the dog and rabbit kidney (McGiff *et al.* 1972; Frame *et al.*, 1974) and newly formed prostaglandin increases juxtamedullary blood flow in the dog kidney under *in vitro* conditions (Itskovitz, Terragno & McGiff, 1974).

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